FOOD MICROBIOLOGY AND FOOD SAFETY

Fidel Toldrá
Editor

Safety of Meat and Processed Meat

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Safety of Meat and Processed Meat

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Preface

The main goal of this book is to provide the reader with the recent developments in the safety of meat and processed meat, from the abattoir along the full processing chain till the final product.

To achieve this goal, the proposal uses five approaches. The first part deals with the main biological contaminants like pathogenic microorganisms, specially *E. coli* and *L. monocytogenes* and toxins, meat spoilage and BSE material that can be present in either meat or its derived products. The second part is focused on main technologies for meat decontamination as well as other developments like active packaging or bioprotective cultures to extend the shelf life. The third part is presenting non-biological contaminants and residues in meat and meat products including nitrosamines, PAH, veterinary drugs and environmental compounds. The fourth part deals with current methodologies for the detection of spoilage and pathogen microorganisms, prions and GMOs, and the final part deals with predictive models, risk assessment, regulations on meat safety and other recent trends in the field.

This book, which is written by distinguished international contributors from 18 countries with solid experience and reputation, brings together all the advances in such varied and different safety approaches related to meat. I thank the production team at Springer and wish to express my gratitude to Susan Safren (editor) and David Parsons (editorial assistant) for their kind assistance to this book.

Valencia, Spain Fidel Toldrá, Ph.D.

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Chapter 1
Main Concerns of Pathogenic Microorganisms in Meat

Birgit Nørrung, Jens Kirk Andersen, and Sava Buncic

Introduction

Although various foods can serve as sources of foodborne illness, meat and meat products are important sources of human infections with a variety of foodborne pathogens, i.e. *Salmonella* spp., *Campylobacter jejuni/coli*, *Yersinia enterocolitica,* Verotoxigenic *E. coli* and, to some extent, *Listeria monocytogenes*. All these may be harboured in the gastrointestinal tract of food-producing animals. The most frequent chain of events leading to meat-borne illness involves food animals, which are healthy carriers of the pathogens that are subsequently transferred to humans through production, handling and consumption of meat and meat products. Occurrences of *Salmonella* spp., *C. jejuni/coli, Y. enterocolitica* and Verotoxigenic *E. coli* in fresh red meat vary relatively widely, although most often are between 1 and 10%, depending on a range of factors including the organism, geographical factors, farming and/or meat production practices.

Zoonotic pathogens in foods, including meats, have to be controlled through a complete, continuous farm-to-fork system and should take into account not only the risks but also technical possibilities, consumers' attitude and behaviours, and cost–benefit analysis. However, some aspects of the control system are pathogen specific. Thus some pathogens in meats (e.g. *Salmonella* spp. and *Campylobacter* spp.) are most efficiently controlled by the main interventions applied in the primary production combined with optimisation of the slaughter hygiene. For some others, such as more environmentally ubiquitous *L. monocytogenes*, but also organisms like *Clostridium* spp. and *Staphylococcus aureus*, the main control measures are focused on later stages of the meat chain.

This chapter is not an exhaustive review of meat-borne pathogens but gives an overview of the main microbial risks associated with the meat chain.

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Although it is clear that meat or meat products can be implicated in all of the above zoonotic infections in humans, understanding in quantitative terms of the importance of meat and meat products compared with other types of food, drinking water and environmental exposure is quite limited. Efforts to quantify the (relative) importance of specific food sources and animal reservoirs for human cases of foodborne illness have been named "human illness attribution". Several human illness attribution approaches, and related data, are currently used worldwide including, analytical epidemiology, microbial sub-typing, analysis of outbreak and comparative exposure assessment (Batz et al., 2005; EFSA, 2008).

Salmonella

Within the genus Salmonella, more than 2000 serotypes exist. Although almost all serotypes are regarded as pathogenic *Salmonella Typhimurium* and *S. Enteritidis* cause almost 60–90% of all human cases of salmonellosis. The reason for this is that these serotypes are far the most predominant in food animals. *S. Typhimurium* can be found in pigs, cattle and chickens while *S. Enteritidis* mainly is found in broilers and table-egg-producing hens.

Salmonella is a gram-negative rod and grows within the temperature range 5–46°C. If present in meat products, heat treatment to around 70°C will kill the organism. *Salmonella* is capable of surviving in frozen meat. *Salmonella* can grow in foods with a water activity down to 0.94 (8% salt) but can survive in products with even lower water activities and in addition it survives in dried foods. *Salmonella* is capable of multiplying both under aerobic and anaerobic conditions as well as in modified atmosphere with 20% CO_2. In addition *Salmonella* grows in foods with pH above 4 (ICMSF, 1996).

Salmonella is found in the environments and in the gastrointestinal tract of farmed and wild animals. *Salmonella* is reported in all farm animals, but most frequently in poultry (EFSA, 2007a). In red meat animals, *Salmonella* findings are most frequent in pigs, followed by cattle. For broiler flocks and layer holdings, recent EU-wide systematic surveys have yielded prevalences of 23.7 and 30.8% respectively, with wide variation between member states (EFSA, 2007b). Recent limited surveillance of cattle and sheep within EU member states has typically yielded Salmonella prevalence below 1% of animals.

Salmonella infections of pigs and poultry are often widespread but typically asymptomatic, whilst ruminants, which are less often infected, more often show clinical signs of illness. When animals are infected with *Salmonella*, the organism will be shed with the faeces and spread to other animals, soil, water and crops.

Animals may get infected with *Salmonella* through environmental contamination, from other animals or through contaminated feed. Humans may get infected from other humans, directly from animals or the environment but most plausible the majority of human cases are caused through contaminated foods.

According to human outbreak data in the EU, eggs and egg products are the foods most commonly implicated in human salmonellosis. Meat especially poultry and pork meats are also commonly involved. Outbreak data collated at the EU level and in many MS do not allow clear identification of meat categories (such as, fresh meat, and products thereof, minced meat and meat preparations) involved in human salmonellosis because food have not been uniformly categorised. In addition, as information is rarely available on food handling and processing practices, it is often not possible to trace back *Salmonella* contamination to the original source (food type) or to deduce the impact of consumer handling. Case–control studies of sporadic cases of salmonellosis have identified the same foods as for outbreaks, as well as several non-food related factors. Source attribution through microbial sub-typing in Denmark has identified layers (eggs) as the major source of human salmonellosis. Among the meat producing animals, pigs and broilers are more important reservoirs for human salmonellosis than cattle (Nørrung & Buncic, 2008; EFSA, 2008). Such studies have not been published in other MS. There are differences in serotype distribution in human cases in MS which may be a consequence of differences in serotype distribution and prevalence of *Salmonella* in food animals, differences in animal production, food processing, food preparation and hygiene and different food consumption patterns.

Human salmonellosis is usually characterised by fever, diarrhoea, abdominal pain and nausea. Symptoms are often mild and most infections are self-limiting within a few days. Occasionally, the infection may be more serious with severe dehydration and even death. Salmonellosis has also been associated with chronic sequelae like arthritis. A total of 160,649 cases of human salmonellosis or (34.6/100,000) were reported within the EU in 2006 (EFSA, 2007a). Most cases are found within the age group 0–4 and 5–14 years.

Campylobacter

Campylobacter are gram-negative, motile, rod-shaped bacteria. The most important species of *Campylobacter* are the thermophilic species: *C. jejuni, C. coli* and *C. lari*, the first two species causing almost all (*C. jejuni* ca. 90% and *C. coli* ca. 7%) human disease.

By comparison with other important foodborne pathogens, such as *Salmonella* species, *Campylobacter* spp. seems ill equipped to survive outside an animal host. They require a microaerobic atmosphere (ca. 5% oxygen and 10% carbon dioxide) and multiply only between 30 and 45°C. However, even at 4°C, low-level metabolic activity can be detected, suggesting that cell integrity is maintained (Park, 2002). Jacobs-Reitsma (2000) reviewed published information on survival of *Campylobacter* spp. in foods. They survive poorly in dry or

acid conditions, and in sodium chloride above 2%. Survival in foods is better at chill temperatures than higher (e.g. ambient), and freezing inactivates many, but not all those bacteria present. *Campylobacter* spp. is relatively sensitive to heat and irradiation, and so can readily be inactivated during cooking (ICMSF, 1996). A new finding is that *Campylobacter* can reside within amoebas (Dahlgren et al., 2003) providing a possible explanation of the survival and persistence of *Campylobacter* in water in particular at low temperatures. The importance of this finding in relation to the epidemiology of campylobacteriosis in animals and humans is not yet clear.

Thermophilic *Campylobacter* spp. is widespread in nature (Jones, 2001). The principal reservoirs are the alimentary tracts of wild and domesticated mammals and birds. This implies that thermophilic *Campylobacter* spp., especially *C. jejuni* and *C. coli*, are commonly isolated from water sources, food animals such as poultry, cattle, pigs and sheep, as well as from cats and dogs (Jones, 2001; FAO/WHO, 2002). *C. jejuni* is predominantly associated with poultry, but can also be isolated from cattle, sheep, goats, pigs, dogs and cats, while *C. coli* is predominantly found in pigs, but can also be isolated from poultry, cattle and sheep (Pezzotti et al., 2003). In animals, thermophilic *Campylobacter* seldom cause disease.

With *Campylobacter*, it is important to note that its reported occurrence in red meats (up to few percents) was drastically lower than in raw broiler meat (up to 66%) although the occurrence in red meat animals – particularly pigs (up to 85% herds) – was not that much dissimilar to poultry (up to 85% flocks). The reasons for that discrepancy are not entirely clarified, although they probably include comparably less faecal contamination occurring in red meat abattoir operations and more extensive dying-off of the pathogen on drier surfaces of red meat carcasses.

Campylobacter spp. may be transferred to humans by *direct* contact with contaminated animals or animal carcasses or *indirectly* through the ingestion of contaminated food or drinking water (FAO/WHO, 2002). The contribution of the various food and non-food sources to the incidence of campylobacteriosis within EU will be country and time dependent due to various factors such as climate, consumption patterns, drinking water distribution, food production systems, degree of implementation of control measures, etc. In several countries the foodborne routes are considered to be responsible for the vast majority of cases and poultry meat is regarded as the most commonly implicated food.

The most common symptoms of human Campylobacteriosis include diarrhoea often bloody, abdominal pain, fever, headache and nausea. Usually infections are self-limiting and last a few days but complications such as arthritis and neurological disorders occur occasionally. In 2006 a total of 175,561 cases of campylobacteriosis were reported by 22 EU Member states (MS). The EU incidence of 46.1 per 100,000 population makes campylobacteriosis the most frequently reported zoonotic disease in EU (EFSA, 2007a).

Yersinia

Among the genus *Yersinia*, *Y. enterocolitica* and *Y. pseudotuberculosis* are foodborne pathogens. *Y. enterocolitica* is by far the most frequent cause of yersiniosis worldwide. The main reservoir for *Y. enterocolitica* is pigs. *Y. enterocolitica* occurs in several biotypes and serotypes, which differs in pathogenicity to humans, geographical distribution and animal reservoirs (EFSA, 2007d).

Yersinia is a psychrotrophic gram-negative rod, with a growth potential down to about 0°C. This is the most remarkable feature of Yersinia, and presumably the explanation for the emergence of this pathogen in the beginning of the 1960s. Yersinia has optimum temperature at 25–37°C and may grow up to about 42°C. It is easily killed by heating and has a tolerance to pH, $a_W$and atmosphere comparable to other enterobacteriaceae (ICMSF, 1996).

In Europe, the human pathogenic types of *Y. enterocolitica* are strongly associated with pigs. Pigs are asymptomatic carriers of biotype 4, serotype O:3, which is the most frequently found variant in human cases of yersiniosis. Another variant, biotype 2 (serotypes O:5,27 and O:9), is less frequently found in pigs. This variant is less frequently found as a cause of human yersiniosis in Europe. The organisms are located in the oral cavity and the intestine of pigs in high frequencies, ranging from 25 to 80%. The organism was found in significant frequencies, up to 25%, as on carcass surfaces of healthy slaughter pigs. Improvement of slaughter hygiene, especially with regard to reduction of faecal contamination has been shown the potential to reduce contamination of this organism dramatically (Andersen, 1988; Andersen, Sørensen, & Glensbjerg, 1991; Nesbakken, Eckner, Høidal, & Røtterud, 2003). Other animals (cattle, sheep, goats, deer, dogs, cats and rodents) have less frequently been found to carry human pathogenic Yersinia. Due to contamination from animal reservoirs, the organisms have also been found in the environment, which have caused several human outbreaks.

Y. enterocolitica has been isolated from foods, especially from pork, where up to 30% of minced pork has been found to contain pathogenic strains (Andersen et al., 1991). Classical isolation methods are however laborious, and they have been found to have a low sensitivity, compared to modern DNA-based methods (Lambertz, Granath, Fredriksson-Ahomaa, Johansson, & Danielsson-Tham, 2007). For this reason the prevalence reported on occurrence in foods must be considered to be seriously underestimated (EFSA, 2007d).

Human cases of yersiniosis are greatly varying in severity, from uncomplicated, self-limiting to serious cases requiring hospitalisation. Yersiniosis mainly affects young children. Diarrhoea, which may be bloody, is the most frequent symptom. The enteritis may be located at the last part of ileum, causing a localised pain that may be confused with appendicitis. Secondary complications in the form of aseptic joint inflammations are rather frequent following

infections with *Y. enterocolitica*. These complications can be very severe, to the point of invalidation, and may last for months.

Yersinia is the third most frequent cause of foodborne disease. In 2006, 8,979 confirmed cases of yersiniosis were reported from the EU member states. This represented a decrease from 9,533 cases in 2005, corresponding to 5.8% and a decrease in the community incidence from 2.6 to 2.1 per 100,000 population. The majority of the cases are caused by *Y. enterocolitica*. Only few countries have reported on disease caused by *Y. pseudotuberculosis* (EFSA, 2007d).

Verotoxigenic *Escherichia coli* (VTEC)

VTEC are strains of *E. coli* capable of producing certain cytotoxins. Some of these may also be enterohaemorrhagic, EHEC, due to additional pathogenic factors. Several serotypes of VTEC are known, however the majority of cases of human illness, including outbreaks have been caused by serotype O157. In the last years the proportion of non-O157 infections has been increasing (EFSA, 2007c). VTEC are most commonly found in cattle, but may also be found in several other animals.

VTEC are part of the *E. coli* species. They may grow down to about 8°C, and up to about 45°C, with an optimum at 37°C. They survive for weeks at 5°C, and for months, even years, at –20°C. VTEC have been shown to be remarkably tolerant to acidic environments and survive for prolonged periods in acidic foods as cider, yogurt and fermented sausages. They have no unusual resistance to heat and will not survive pasteurisation (Willshaw, Chaesty, & Smith, 2000; Duffy, Walsh, Blair, & McDowell, 2006).

VTEC are zoonotic pathogens. Cattle are considered to be the main animal reservoir. EFSA reported (2007a) that the majority of findings of O157 were in cattle, with findings up to 13.7%. Other ruminants including sheep and goats may also harbour VTEC and are considered important reservoirs. Pigs may be infected with VTEC but are not considered a major reservoir. VTEC rarely cause disease in animals (EFSA, 2007c).

Undercooked meat and meat products have on several occasions been involved in human disease. Also unpasteurised dairy products have been a source of infection. Acidic foods, fermented sausages, yogurt and cider have been involved in several outbreaks. Drinking water has also been involved in outbreaks, due to spread of faecal contamination of the environment. In investigations in the EU member states VTEC was found in cattle meat (up to 7.2%) pig meat (up to19.7%) in meet from sheep (0.7–11.1%) and in raw cow's milk, and cheeses made from unpasteurised milk (up to 16.2%) (EFSA, 2007a).

Infections are characterised by diarrhoea that vary from mild to severe, bloody and painful. In about 10% of the cases patients develop severe complications, haemolytic uraemic syndrome (HUS), characterised by acute renal

failure and anaemia, which may be fatal. HUS is mainly observed in children. A total of 4,916 cases (1.1 cases per 100,000 population) were reported in 2006 from the EU member states (EFSA, 2007c).

Listeria

The genus Listeria consists of six different species but only *L. monocytogenes* is regarded as pathogenic to humans. *L. monocytogenes* is a gram-positive rod and grows within the temperature range –0.4–45°C, with an optimum at 37°C. Thus *L. monocytogenes* is capable of multiplying in refrigerated foods including meats. If present in meat products, heat treatment to around 75°C will kill the organism. *L. monocytogenes* can grow in meats with a water activity at 0.92 and may survive in products with even lower water activities. *L. monocytogenes* is capable of multiplying both under aerobic and anaerobic conditions and thus can multiply in vacuum-packed meat and meat products. In addition *L. monocytogenes* grows within a wide pH range between pH around 4.6 and 9.4 (ICMSF, 1996). In laboratory media under optimal temperatures between 20 and 30 degrees and prolonged incubation they are found to be able to multiply even at pH values around 4.1°C.

L. monocytogenes occurs widely spread in nature in wild animals, plants, as well as in the soil. Farm animals, especially ruminants may be healthy carriers of *L. monocytogenes,* which can be found in the faeces of the animals with different prevalences (from few percent to around 50% of the animals in different studies). Animals and especially ruminants can also contract listeriosis and improperly fermented silage has been found to be the source of listeriosis in livestock.

Healthy ruminants but also poultry and pigs will from time to time habour *L. monocytogenes* in the gastrointestinal tract when brought to the slaughterhouse.

L. monocytogenes is very often found to colonise the meat production environment and thus is often isolated in samples from production rooms and refrigerating rooms in slaughterhouses. Because of this and the ubiquitously nature of the organism a wide range of different foods including both raw and ready-to-eat meat can be contaminated with *L. monocytogenes*. However for healthy human population, only ready-to-eat foods including meat that contains high numbers (more than 100–1,000 cfu/g) is considered to pose a risk. For these reasons monitoring of this pathogen is focused primarily in ready-to-eat (RTE) foods including RTE meats.

In a US risk assessment (USDA, 2003) deli-meats and frankfurters were found to be the foods with the highest risk/serving of causing human listeriosis.

In 2006, 23 MS reported a large number of investigations from foodstuffs. The proportion of the samples exceeding the legal safety criterion of 100 *L. monocytogenes* colony forming units (cfu) per gram was most often observed in ready-to-eat (RTE) fishery products (1.7%), followed by cheeses (0.1–0.6%),

other RTE products (0.1–0.4%) and RTE meat products (0.1%) at the EU level.

Listeriosis is the disease caused by infection with *L. monocytogenes*. Symptoms of listeriosis may range from mild flu-like symptoms and diarrhea to life threatening forms characterised by septicaemia and meningitis. In pregnant women, the infection may spread to the foetus and result in abortion or birth of a child with septicaemia. The incubation period is rather long from 5 to 70 days for invasive listeriosis while it is only about 24 h for febrile gastroenteritis. Human listeriosis is rare but the invasive form, septicaemia and meningitis, is severe. Old and immunocompromised persons are those most often affected. In 2006, 1,583 cases of listeriosis were reported from the 25 MS in the EU, with 56% of cases occurring in individuals above 65 years of age (EFSA, 2007a). The overall incidence was 0.3 cases per 100,000 population.

Staphylococcus aureus

S. aureus is a pathogenic microorganism that may cause infections as well as foodborne intoxications. It is a gram-positive rod with a remarkable resistance in environments.

S. aureus will grow in the temperature interval 7–48°C, with optimum temperature around 37°C. Production of enterotoxin can occur between 10 and 48°C. They are easily killed by heating. Staphylococci are the most salt resistant of pathogenic microorganisms, growing in salt concentrations up to 15%. They survive well in dry environments. Staphylococci are facultative anaerobic microorganisms but grow faster in aerobic conditions. Staphylococci grow at pH between 4 and 9. Staphylococci are reported to be poor competitors as they grow poorly in mixed cultures (ICMSF, 1996; Baird-Parker, 2000).

Staphylococci are naturally present on the skin and mucous membranes on animals, including healthy animals that are brought for slaughter. Furthermore Staphylococci are potential pathogens that may be present in various pathological conditions, i.e. in abscesses, skin inflammations and purulent processes in a variety of organs. Also Staphylococci are a natural inhabitant of personnel, which provides a source for contamination. It has been shown that 20–30% of the population are permanent carriers of staphylococci, whereas 60% of the population are intermittent carriers (Klytmans & Wertheim, 2005). It has been found that the prevalence of enterotoxin production is greater among isolates from human carriers as compared to isolates from foods (Lawrynowiez-Paciorek, Kockman, Piekarska, Grochowska, & Windyga, 2007). ICMSF (1998) states that a low contamination of the carcass and fresh meat during slaughter is unavoidable, which is supported by base-line studies performed by USDA (1996), that revealed findings of an average of 84 CFU *S. aureus* per cm^2, based on an examination of 2,100 samples from chilled pig carcass halves.

In fresh meat this potential contamination is of little consequence. A psychrotrophic flora dominates the microbial flora on-meat, gram-negative bacteria that presents effective barrier for staphylococci, which is described as a poor competitor. Also as fresh meat is kept refrigerated, growth of staphylococci is not possible.

However, if contaminated meat is subjected to conditions that inhibits the competing flora, and at the same time favour growth of staphylococci, they may present a risk for the consumer. Salting inhibits the psychotropic flora, and smoking and curing may bring the meat into the temperatures that allow growth and formation of enterotoxin. Meat products that are salted, smoked and cured at elevated temperatures have on occasions been reported to be a source of staphylococcal enterotoxin poisoning (Nychas & Arkoudelos, 1990). It has been reported though that even during natural fermentation the naturally occurring staphylococci are controlled by the intrinsic microbial flora (Aquilanti et al., 2007). In most modern production starter cultures are used, increasing the effectiveness of fermentation, especially in the early phase, thereby further increasing the safety of the production (Niskanen & Nurmi, 1976; Meisel, Gehlen, Fischer, & Hammes, 1989). Control of the physical parameters for the processing will also increase the safety of salted, cured and fermented products (Unterman & Mueller, 1992).

Staphylococcal food poisoning is characterised by a short incubation time, dramatic course and short duration. Few hours after ingestion of the contaminated food the patient will feel nausea, which develops into violent vomiting. Stomach pains and diarrhoea follow. The patient will often get a strong headache. Normally the disease dissolves quickly, typically within 24–48 h.

Staphylococcal enterotoxin is formed during the growth of the bacterium in the food. When the food is ingested, the toxin that is already formed in the food is absorbed through the stomach wall. The symptoms therefore occur shortly, within hours, after ingestion of the contaminated food. The toxin affects the central nervous system and causes the patient to vomit.

Only a fraction of staphylococcus strains are able to produce enterotoxin. Rosec, Guiraud, Dalet, and Richard (1997) found that 77 of 213 strains isolated from foods were able to produce enterotoxins. However, another investigation reported that of 106 strains isolated from bovine mastitis, none were found to produce enterotoxins (Aarestrup, Andersen, & Jensen, 1995).

The typical scenario for staphylococcal food poisoning is by contamination of a heat-treated food, through handling by personnel, followed by a temperature abuse. Heating will destroy most of the competing flora, which together with cooling failure will provide ideal conditions for growth of staphylococci, should the food by accident or malpractice be contaminated.

Sufficient amounts of enterotoxin to cause food poisoning require that *S. aureus* have been growing to relatively high numbers in the food, about 10^6 (Niskanen & Nurmi, 1976; Otero, García, García, Moreno, & Bergdoll, 1990; ICMSF, 1998).

Clostridia

Clostridium perfringens and *C. botulinum* are potentially pathogenic microorganisms that are often contaminants in fresh meat. They are strictly anaerobic bacteria that may be present in the normal gut flora of animals and humans. They are spore-forming bacteria enabling them to survive in unfavourable environments, which present a challenge in food preservation.

C. perfringens will grow in the temperature range from 15 to 50°C. At optimum temperature, 35–40°C, the growth rate is very fast, with a generation time down to about 7 min. The organism requires a high water activity for growth. It will not grow at a_w below 0.97. The spores can survive cooking for several hours (ICMSF, 1996).

The species *C. botulinum* are defined by a Clostridium, able to produce botulinum toxin. Therefore *C. botulinum* comprises of a varied group of types, which differs in growth requirements for temperature, water activity, pH and heat treatment necessary for inactivation. They also differ in metabolism and geographical distribution. The psychrotrophic group has an optimum temperature at 28–30°C, will grow down to 3.3°C and endures water activity down to 0.97 (5% salt) and pH 5.0. The mesophilic group has an optimum at 35–40°C, grows down to 10–12°C and endures water activity down to 0.94 (10% salt) and pH down to 4.6 (ICMSF, 1996).

C. difficile has in recent years demanded increased attention. This bacterium may cause enteritis in patients, especially in association with antibiotic treatment. *C. difficile* is occurring in domestic animals, in pets as well as food animals, where it may also cause infection (Songer, 2004). DNA-typing techniques suggest that animals may be a potential source of infection of humans (Rodrigues-Palacios et al., 2006).

Clostridia are naturally occurring in soil and water, and often found in the gut of humans and animals. They will be present in raw meat in low numbers, depending on the level of hygiene at the abattoir. An overview of prevalences of *C. botulinum* was presented by Lund and Peck (2000). Insufficient heating and inadequate refrigeration of foods will contribute to spore survival, growth and in case of *C. botulinum* to toxin production. Consumption of such a food, without prior heating, will constitute a risk of poisoning with either perfringens-enterotoxin, or botulinum toxin. Sufficient heating will kill vegetative cells of *C. perfringens*, and destroy botulinum toxin. Botulinum toxins are sensitive to heat treatment. Toxins are rapidly destroyed at 75–80°C (Labbè, 2000; Lund & Peck, 2000).

C. perfringens poisoning are one of the most common foodborne disease, however presumably with most cases never recorded because of mild and self-limiting disease.

Cases of botulinum are rare, but serious. Hauschild (1989) presented an extensive overview of outbreaks of botulism worldwide. More recent reports

from the United States and the European Union have been presented by Sobel, Tucker, Sulka, McLaughlin, and Maslanka (2004) and Therre (1999).

C. botulinum poisoning, botulism, is characterised by neurological symptoms: disturbed vision, dry mouth, difficulty in speech and swallowing and progressing paralysis of muscles, including respiratory muscles and the heart, finally causing death. Vomiting and diarrhea may be part of the symptoms. The incubation time is wide, from 2 hours up to 8 days. The fatality has previously been very high but has today been reduced significantly.

C. perfringens poisoning is caused by an ingestion of a large amount of vegetative bacteria. In the first part of the gut, during sporulation, enterotoxin is released in the gut causing diarrhea, sometimes accompanied by stomach cramps, but usually mild and self-limiting. Symptoms occur 8–24 h after ingestion of the meal.

Clostridia, like Staphylococci and Bacilli is organisms that are naturally present in the environment. Improvement of good hygienic practices will contribute to reduce the levels in raw foods, including meat, but cannot eliminate these organisms.

Control of Microbial Foodborne Pathogens in the Meat Chain

Occurrence of Foodborne Pathogens in the Meat Chain

The original sources of the main foodborne pathogens are asymptomatic farm animals excreting the pathogens in their faeces. Subsequently, further spread of pathogens on-farm and along the meat production chain occurs via various direct or indirect routes due to faecal contamination. This ultimately results in human exposure to those pathogens via meats/foods. Recently reported data on the occurrence of the main microbial foodborne pathogens obtained from the three main phases of the meat chain, i.e. pre-harvest (farm animals), harvest (raw meats) and post-harvest (ready-to-eat meats) in the EU are indicated in Table 1.1.

Although the reported occurrences of these microbial pathogens in different animal species and related meats varied considerably between individual EU countries, overall, it could be assumed that *Campylobacter* spp. and *Salmonella* were most frequently associated with the poultry meat chain, whilst VTEC with the beef chain. Reported data on *L. monocytogenes* in animals and raw meats were scarce, but a large amount of data on this pathogen in RTE foods is available – the pathogen was most frequently associated with RTE from pork. Furthermore, the presented data confirm that microbial foodborne pathogens can be present at multiple points of the meat chain; therefore, meat safety assurance must be based on control measures implemented at all those points.

Table 1.1 Reported occurrence of the main microbial foodborne pathogens in the meat chain in the European Union in 2005 (Adapted from Nørrung & Buncic, 2008)

	Reported occurrence (%)			
Sampling point	*Campylobacter*	*Salmonella spp.*	VTEC	*Listeria*
Cattle on farm*	0.3–46.9	0–6.7	*0–21.6*	DNA/I
Raw beef	0–2.1	0–8.3†	*1–7.1*†	DNA/I
Bovine RTE	DNA/I	DNA/I	DNA/I	0.7–5.3
Pigs on farm*	24.7–85.4	0–60.0	0–9.2	DNA/I
Raw pork	0–0.5	0–18†	0–6.2	DNA/I
Porcine RTE	DNA/I	DNA/I	DNA/I	*0–26.5*
Sheep/goats*	DNA/I	DNA/I	0–11.8 (FD)	DNA/I
Raw meat	DNA/I	DNA/I	0 (FD)	DNA/I
Ovine/caprine RTE	DNA/I	DNA/I	DNA/I	DNA/I
Poultry**	*0.2–85.2*	*0–18.2*	DNA/I	DNA/I
Raw poultry	*3.1–66.4*	*3.9–18.5*	DNA/I	DNA/I
Poultry RTE	DNA/I	DNA/I	DNA/I	0–3.1

* Herds; ** Flocks; † Including minced meat; *In italics* – highest overall occurrence of a given pathogen
RTE – Ready-to-eat food; FD – Few data; DNA/I – Data not available or insufficient

Meat Safety at Pre-harvest Level

Although in-depth consideration of the risk factors and the controls on-farm would need to be both pathogen- and animal species-specific, the main principles of and measures for control of foodborne pathogens on-farm are common and applicable relatively universally. These are summarised in Fig. 1.1.

Prevention of Recycling of Pathogens in the Environment

Farm animals can be exposed to microbial pathogens through grazing, harvested feed or a water supply that has previously been contaminated by

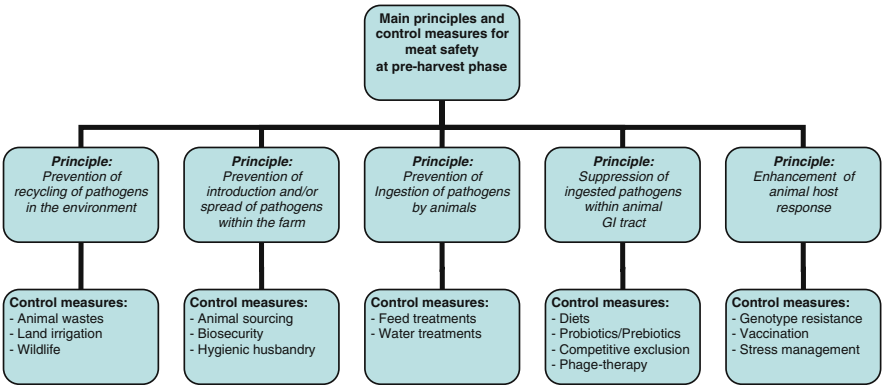

Fig 1.1 Main principles and control measures for meat safety at pre-harvest phase

spreading untreated abattoir- and/or farm wastes (manure, slurry) containing enteric pathogens as fertilizers on agricultural land (Pepperell et al., 2005; Hutchison, Walters, Avery, & Moore, 2004). Therefore, inappropriate land management can mediate infections and/or re-infections of animals with enteric pathogens.

The main control measures to break such an animal population–environment–animal population cycle of microbial pathogens include appropriate storage of manure and "lagoon" treatment of effluents before their application on land. Manure "composting" eliminates a very large proportion of microbial pathogens through generation of heat of 55–60°C leading to "auto-sterilisation" (Hutchison, Nicholson, Smith, Keevil, & Moore, 2000).

Prevention of Introduction and/or Spread of Pathogens Within the Farm

Infected, newly introduced animals are one of the most important sources of foodborne pathogens on farms. This can be prevented by purchasing animals only from controlled sources. Connected with this, it is known that the so-called "all in-all out" farming system with effective sanitation between the animal lots is an effective measure reducing the "farm infection" risks. Furthermore, introduction of microbial pathogens into, as well as spread of pathogens within, the farm can occur through various vectors including infected wildlife and/or vermin, infected or contaminated farm staff/visitors and contaminated farm equipment.

Therefore, implementation of effective biosecurity system to prevent/minimise exposure of the animal population to these vectors must be ensured. Once introduced to the farm environment, pathogens can survive days to months in/on various substrates: faeces, soil, water and building materials (Hutchison et al., 2000). Generally, regardless of the animal-related substrate, pathogens such as *Salmonella*, *Campylobacter* and *E. coli* O157 survive better under dirty/humid/cold than under clean/dry/warm environmental conditions (Small, Reid, & Buncic, 2003). It is of interest that significant strain-related variability in the survival can exist, as shown with *E. coli* O157 (Avery & Buncic, 2003).

Within infected farms, it is known that proximity of animals associated with intensive indoor farming (i.e. group housing) contributes to increased horizontal transmission of pathogens, compared to outdoor farming. Possible routes for pathogens' spread include contaminated aerosols, frequent physical contacts with contaminated environmental surfaces or contaminated animal coats in a confined space and social exchanges (licking/grooming). In addition, animal feeders and water drinkers, when used by more than one animal, can serve as important sources of cross-contamination and animal infections. Overall, the implementation of hygienic animal husbandry practices including effective cleaning/sanitation regimes is one of crucial aspects of on-farm meat safety assurance.

Prevention of Ingestion of Pathogens by Animals

Contaminated animal feeds are an important source of foodborne pathogens ingested by animals. This has been particularly proven by, but is not limited to, examples of *Salmonella* spp. in poultry and pigs. In addition, contaminated drinking water can be another source of pathogens' ingested by animals. With respect to animal feeds, two sources of microbial contamination are of particular concern: the proteinaceous ingredients included during its preparation and vermin (rodents, birds) contacting them during its further handling/storage.

Therefore, the purchase of feeds from controlled sources and their vermin-proof storage until use are important elements of the on-farm meat safety system. Interestingly, the strains of pathogens present in purchased animal feeds and those that predominate in animal population do not necessarily correlate. For example, strains of *Salmonella* spp. associated with purchased feed are often transient ("exotic" strains), whilst some other strains of *Salmonella* spp. are often more persistent and well established on-farm ("local" strains).

In any case, to prevent/reduce ingestion of pathogens by animals on-farm, feed can be subjected to some antimicrobial treatments shown to be beneficial, e.g. in *Salmonella* control in pigs, including: fermentation (liquid feeds), acidification by acidulants and heat treatments. Antimicrobial treatment of drinking water is another measure to reduce the risks.

Suppression of Pathogens Within Animal Gastrointestinal Tracts

Results from some experimental studies indicated that the occurrence and/or levels of shedding of the pathogens by farm animals can be reduced by some dietary manipulations. However, overall, the actual relevance of particular diets for faecal shedding of foodborne pathogens – e.g. whether shedding of *E. coli* O157 is higher in grain-fed or hay-fed cattle – is still unclear. There is little doubt that direct comparison of results from different studies on the effects of given diets on the shedding of pathogens from a given animal species is very difficult due to interference of other animal- and/or farm-related variable factors acting simultaneously.

Furthermore, animals can be fed with viable microorganisms antagonistic towards pathogens via either modifying environmental factors in the gut or producing certain antimicrobial compounds – the so-called "probiotic" strategy (Fuller, 1989).

Also, selected nutrients can be fed to animals enabling the normal gut microflora to expand its role and enhance its competitiveness – the so-called "prebiotic" strategy. Those nutrients (sugars or other organic compounds) are not digestible by the animal; instead, they are utilised by some commensal microorganisms in the gut (Walker & Duffy, 1998; Steer, Carpenter, Tuohy, & Gibson, 2000).

Moreover, feeding animals with selected, non pathogenic bacterial strains can lead to reduced attachment of pathogens to the gut mucosa – the so-called "competitive exclusion" strategy (Nurmi et al., 1992). Depending on the maturity of the animal gut, this approach can be aimed at exclusion of pathogens from the naïve gut of a neonatal animal, or the displacement of an already established pathogenic bacterial population. The competitive exclusion concept is applied primarily in monogastric animals, e.g. poultry and pigs; it has been demonstrated that *Salmonella* colonisation in intensively reared chicks can be inhibited by feeding them with gut content of mature hens.

Also, bacteriophages have been used to control foodborne pathogenic bacteria in farm animals, e.g. *E. coli* O157 in a sheep model (Kudva, Jelacic, Tarr, Youderian, & Hovde, 1999). Nevertheless, the actual effectiveness of phage treatment under practical conditions has been variable.

Although addition of antibiotics to animal feed for the purpose of controlling microbial (including foodborne) pathogens has been advocated in some circles and some countries, this approach can have negative effects on animal health and lead to the spread of antibiotic resistance (Witte, 2000) so is discouraged in the EU.

Enhancement of Animal Host Response

Vaccination of animals, particularly when combined with other measures implemented further along the food chain, is considered as a promising strategy for foodborne pathogen reduction. For example, in pigs and cattle, vaccines against *Salmonella* strains causing disease have been successfully used (House et al., 2001); and vaccination of poultry against *Salmonella* contributed to significant reduction of the pathogen in poultry meat in the UK. Nevertheless, for other pathogens, such as *E. coli* O157 or *Campylobacter*, vaccines are being researched but effective ones are not yet commercially available.

In addition, it is known that stress in animals can disturb the normal, balanced gut microflora in farm animals resulting in reduced resistance against colonisation with pathogens, e.g. *Salmonella* spp., weakened immune responsiveness and increased shedding of pathogens. Some stressors occur "naturally", e.g. parturition and weaning, whilst others occur due to poor animal husbandry, e.g. inadequate housing, sudden changes in diet and rough handling. Therefore, stress management is an important aspect of on-farm meat safety assurance.

Meat safety at Harvest Level

In the context of this text, the harvest phase of the meat chain starts with transport (as it is most often managed by abattoirs) and includes lairaging, slaughter and dressing of animals, and ends with obtaining raw meats during

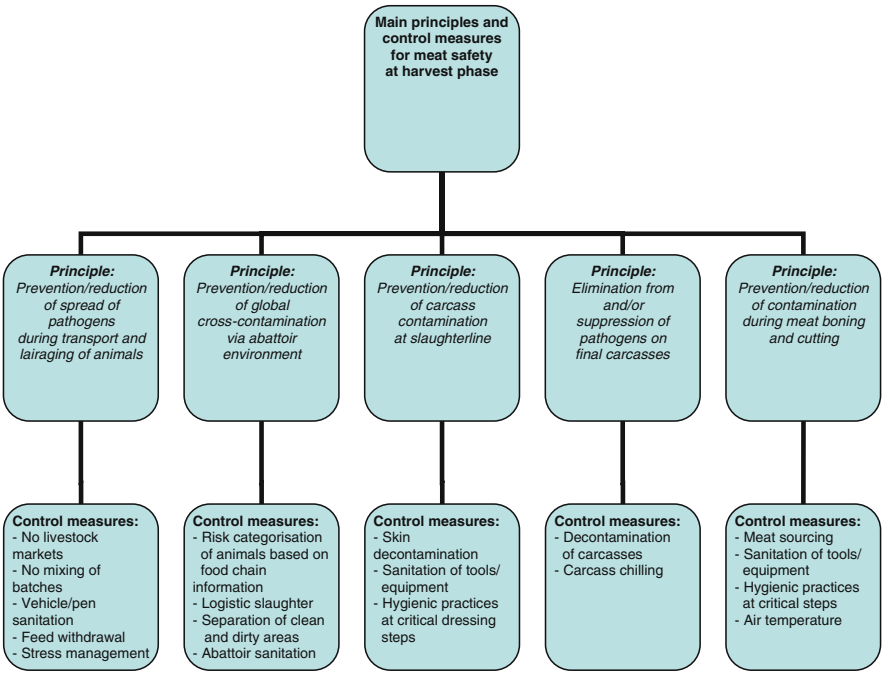

Fig 1.2 Main principles and control measures for meat safety at harvest phase

boning/cutting operations. The main principles of, and measures for, controlling foodborne pathogens at harvest level are summarised in Fig. 1.2.

Prevention/Reduction of Pathogen Spread During Transport and Lairaging

Transport and lairaging (T–L) lead to increased faecal shedding and/or levels of foodborne pathogens in animals (Berends, Urling, Snijders, & Van Knapen, 1996; Fravalo, Rose, Eveno, Salvat, & Madec, 1999). This can be caused by stress-mediated reactivation of on-farm latent infection, so ensuring animal welfare during T–L is a relevant meat safety measure.

Furthermore, microbial cross-contamination via animal-to-animal and/or animal-to surfaces-to animal routes occurs during T–L and, similarly, in livestock markets (Collis et al., 2004). Consequently, preventing mixing of different batches of animals and avoiding inclusion of livestock markets within the T–L phase reduce foodborne pathogens in animals at slaughter. Also, effective sanitation of vehicles and pens is essential to reduce T–L-related cross-contamination, but naturally occurring pathogens (e.g. *Salmonella*, *E. coli* O157) can persist on surfaces even after routine sanitation (Small et al., 2003; Tutenel, Pierard, Van Hoof, & De Zutter, 2003). Overall, it can be assumed that the shorter the T–L duration, the lesser the spread of pathogens among animals.

Also, the use of feed withdrawal as a means to reduce amount and excretion of faeces and related microbial contamination in animals during T–L (and slaughterline) operations has been studied. Whilst some beneficial effects may be achievable in the case of poultry, fasting in some other animals species can actually increase pathogen shedding, e.g. *E. coli* O157 and *Salmonella* in cattle (Cray, Casey, Bosworth, & Rasmussen, 1998; Delazari, Iaria, Riemann, Cliver, & Jothikumar, 1998).

Prevention/Reduction of Global Cross-Contamination via Abattoir Environment

Incoming live animals, carrying pathogens on their surface (skin/feathers) and within their guts, serve as a source of global contamination of the abattoir environment. Once contaminated, the abattoir environment can serve as a source of cross-contamination of subsequently handled animals and their carcasses, even if they were originally pathogen free. Through analysis of information on the pre-harvest history of incoming animals, i.e. the so-called "Food Chain Information" (FCI), batches of animals can be ranked according to the risk they pose as a source of zoonotic agents including microbial foodborne pathogens. Relevant FCI information includes visual cleanliness of animals, animal movement records, epidemiological intelligence data including those from herd health plans and monitoring/surveillance; as well as farm management/QA data including those on land management, feeds, biosecurity and animal husbandry.

Based on FCI, batches of animals from the lower-risk category with respect to pathogens can be slaughtered either in separate abattoirs or separately (before) the higher-risk batches in the same abattoirs. This approach is known as "logistic" slaughter (Swanenburg, van der Wolf, Urlings, Snijders, & van Knapen, 2001).

Strict separation between dirty (e.g. the lairage area) and clean (e.g. the slaughter-hall) zones including separate flow of staff, equipment and airflow further reduce global abattoir environment-mediated microbial cross-contamination in abattoirs.

Prevention/Reduction of Carcass Contamination on the Slaughterline

The occurrence of microbial pathogens on animal skins and dressed carcasses can be significantly correlated as demonstrated, for example, with *E. coli* O157 in slaughtered cattle (Elder et al., 2000). Potential approaches to reducing the animal coat-mediated microbial carcass contamination include hide decontamination after slaughter but before skinning of cattle (Small et al., 2003; Koohmaraie et al., 2005); this is used in commercial abattoirs in the USA but not in the EU presently. It seems that the reductions in microbial and foodborne pathogens' counts on hides achievable by various treatments (e.g. singeing, commercial sanitisers or disinfectants, organic acids, electrolysed water) are,

most commonly, around 2–3 log reduction levels under commercial abattoir conditions.

During dressing of slaughtered animals, the hygienic practices at critical steps differ fundamentally between abattoirs' technologies used for different meat animal species (cattle, sheep, pigs and poultry). Furthermore, variations in technology and process hygiene can be marked even between abattoirs slaughtering the same species. The critical dressing steps from the perspective of carcass contamination with microbial pathogens in cattle/sheep abattoirs are similar (e.g. skinning, evisceration, chilling) whilst in pig and poultry abattoirs they include some others, such as scalding.

The main control measures to prevent/minimise carcass contamination with pathogens include prevention of (a) contact between animal coats and carcass meat; (b) content spillage from gastrointestinal and/or genital tracts; and (c) contamination via physical "vectors" such as scalding water, aerosols and tools/equipment.

In particular, to avoid transmission of pathogens from one carcass to other, all tools/equipment coming in contact with edible tissues must be "sterilised" between individual carcasses.

Elimination from and/or Suppression of Pathogens on Final Carcasses

Total prevention of microbial contamination of beef carcasses solely by process hygiene is unachievable under commercial abattoir conditions. For that reason, and because regulators and/or producers want to reduce the microbiological risks further, the use of decontamination treatments of dressed (final) carcasses has attracted much attention. In the USA, dressed carcass decontamination is used routinely, whilst presently it is not used routinely in the EU. The Article 3(2) of the EU Regulation 853/2004 provides a legal basis for "...the use of substances other than potable water to remove surface contamination from foods of animal origin" including meat. A decontamination agent is considered as "processing aid" if removed following the application. If it is not removed, it is considered as a "food additive" (e.g. preservatives, glazing agents) and its approval for meat follows Directive 89/107/EEC.

Meat decontamination includes treatments with steam/hot water achieving on-meat temperatures between 80°C and 85°C or with organic acid- or non-acid chemical solutions (Smulders & Greer, 1998; Sofos & Smith, 1998). It is important to note that, to date, decontamination treatments only proportionally reduce the microbial load from meat; up to 2–3 logs microbial reductions are achievable under commercial abattoir conditions. Higher microbial reductions (up to 3–4 logs) can be achieved through sequential use of two or more decontamination treatments (Graves Delmore, Sofos, Schmidt, & Smith, 1998). Overall, carcass decontamination should not be considered as a substitute for – but only as an addition to – good hygienic practice.

In addition, the carcass temperature immediately after slaughter can increase from around 37°C to around 40°C due to intensive post-mortem biochemical

activities in the pre-rigour muscle. This carries a risk of proliferation of pathogens on warm meat, so rapid refrigeration of carcasses – commonly to <7°C – is required, which inhibits growth of most enteric pathogens.

Prevention/Reduction of Contamination During Meat Boning/Cutting

Final carcasses (commonly after chilling, sometimes before that) are cut into different parts. In meat cutting plants and raw meat re-packaging centres, the main source of contamination with foodborne pathogens is likely to be incoming meat. Subsequently, the contamination is spread onto freshly cut surfaces of meat through intensive manipulation and handling of meat via working surfaces, conveyers, hands and tools/equipment.

Therefore, the staff has to be properly trained in the application of hygienic working practices at critical steps of the operation. Furthermore, an effective regime of cleaning/sanitation of the surfaces/equipment – preferably repeatedly applied during the day – has to be implemented.

To suppress the microbial growth on both the handled meat and the surfaces, the air temperature in the boning/cutting rooms is maintained at ≤12°C; further refrigeration of cut meat during its storage also must be ensured.

Meat Safety at Post-Harvest Level

Further Processing of Meat

Generally, meat processing techniques can involve various treatments including salting (addition of sodium chloride), curing (addition of sodium chloride plus other additives, e.g. sodium nitrite, potassium nitrate or polyphosphate), smoking, drying, fermentation and/or heat treatment – applied alone or in combinations. A very large number of different types of meat products exist in different countries. It is not possible to consider them individually here but they can be grouped into several main types based on production technologies and inherent antimicrobial factors (Fig. 1.3).

The behaviour of microbial foodborne pathogens in/on processed meat products depends on three main aspects: (a) genotypic/phenotypic characteristics of the pathogen; (b) intrinsic factors of the product which can affect the pathogens; and (c) extrinsic factors which can affect the product and the pathogens. The ultimate fate of any given pathogen in/on any given processed meat is determined by the combination of these factors and their inter-relationship. The general competitiveness of the main foodborne pathogens with respect to the main factors acting in meat products is illustrated in Table 1.2.

In uncured, mildly heated meats that are often eaten undercooked (e.g. burgers) or practically rare (e.g. beef steak), only non-bactericidal treatments (40–60°C for short time) occur in the meat centre. Microbial pathogens

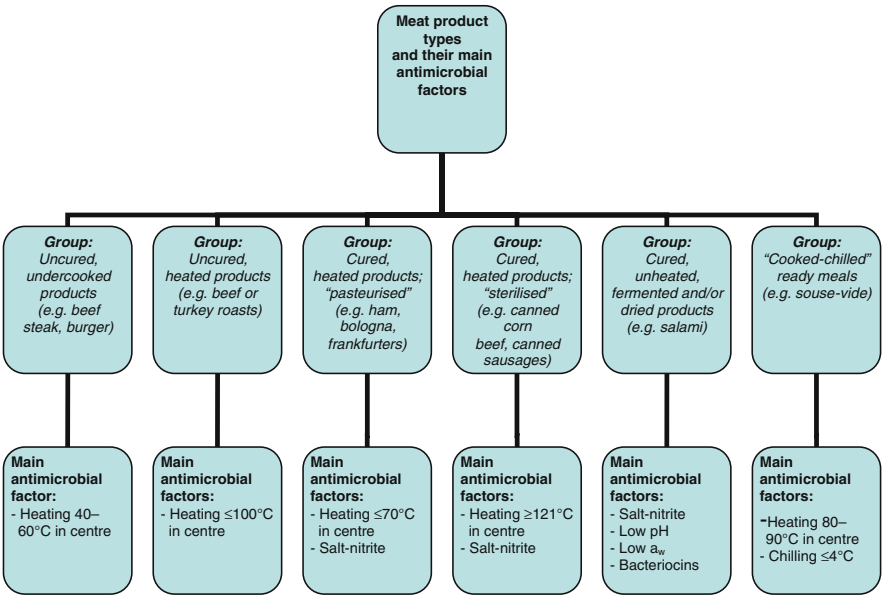

Fig 1.3 Meat product types and their main antimicrobial factors

contaminating the raw meat, e.g. *E.coli* O157 and *Salmonella* are the greatest hazards associated with such products.

In uncured, cooked meats such as roasts in which the temperature reaches only below 100°C in the centre, *C. perfringens* is of the most concern due to the ability of its spores to survive the heat treatment, germinate and proliferate

Table 1.2 General indication of the competitiveness of main foodborne pathogens in processed meats

Foodborne pathogen	Ability to grow at refrigeration (<7°C)	Ability to grow at lower pH	Ability to grow at lower water activity	Ability to survive cooking at 71°C	Probability of post-processing contamination (from processing environment/ staff)
Salmonella spp.	– (+ SS)	+	+	–	–/+
Campylobacter jejuni/coli	–	–	–	–	–/+
Listeria monocytogenes	+ + +	+	+ +	–	+ + + / + +
Escherichia coli O157:H7	–	+ +	–	–	–/–
Clostridium botulinum	– P + + NP	+	+ P – NP	+ + + (spores)	–/–

P – Proteolytic types; NP – Non-proteolytic types; SS – Some strains

during post-cooking handling. Related outbreaks occur only after post-cooking temperature abuse. There are no accompanying spoilage species to make the meat inedible or "warn" consumers. Furthermore, *S. aureus* producing heat-stable enterotoxin before cooking, as well as *L. monocytogenes* and *Salmonella* in case of post-cooking contamination, is associated meat safety concerns in these products.

In cured and heated meats in which the temperature reaches <70°C ("pasteurised" products) such as cooked ham or emulsion-type of sausages, the greatest hazards are the frequent post-processing contaminants *L. monocytogenes* and *S. aureus*.Both rarely survive a proper heat treatment, but their growth is not controlled by the usual concentrations of salt and nitrite in these products.

In cured and heated meats in which the temperature reaches 121°C ("sterilised" or "botulinum-cooked" products) such as canned corned beef or canned sausages, potential hazards include survival of heat-resistant spores or, only in case of post-process leakage, also vegetative forms of other microbial pathogens. In low-acid canned meats, *C. botulinum* is the organism of most concern.

In cured, unheated (raw) fermented and/or dried products such as salami or country-style ham, the usual salt concentrations (even when only 2%) can inhibit some bacteria but not salt-tolerant pathogens, e.g. *L. monocytogenes* and *S. aureus*. Furthermore, nitrites pose a significant inhibitory potential, particularly in fermented meats, as a drop of one pH unit enhances the effect of nitrites 10-fold. On the other hand, water activity is an important factor in dried products. When the products have a higher a_w, *L. monocytogenes*, *Salmonella* and *S. aureus* may proliferate and are the most significant hazards. When they have low a_w, production of *S. aureus* enterotoxin (e.g. in smaller-diameter fermented sausages) or aflatoxin B_1 (e.g. in country hams heavily contaminated with moulds) could occur.

In cooked-chilled ready meals such as "sous-vide", which are part-cooked then vacuum-packed followed by in-pack heat treatment (80–90°C; "pasteurisation") and chilled storage (≤4°C), aerobic microorganisms are suppressed but these products are considered as potentially hazardous with respect to *C. botulinum.*

In meat processing, relying only on the excessive action of a sole antimicrobial factor to achieve safe product has numerous problems. These include detrimental effects on sensory qualities of the product; negative reactions from consumers opposing "unnatural" methods of food preservation; and induction of stress reactions in pathogens that may enhance their resistance or virulence. In modern food technology, therefore, efforts are focused on achieving safety through intelligent combinations of multiple antimicrobial factors – the so-called "hurdle" concept.

Overall, principles for meat safety control measures during meat processing include application of a bactericidal step (heating) wherever possible; prevention of cross-contamination during post-cooking manipulation/handling (e.g. slicing, packaging) from the environment, equipment and staff; inclusion of post-processing pasteurisation of sealed ("in-bag") product; and final product refrigeration where necessary.

Meat Products at Catering-Consumer Levels

Basic principles of microbial meat safety at this level are similar not only for different meat products but also for other foods. The main control measures to reduce the risks from foodborne pathogens include adequate refrigeration, proper cooking, prevention of cross-contamination from raw to ready-to-eat foods (via contaminated refrigerators, hands, cutting boards, knives and kitchen towels) and adequate post-cooking handling including rapid cooling to refrigeration temperature or keeping food at >60°C. Furthermore, it is important that the main principles for safe food preparation are included in educational programs targeting both operators and consumers. For example, based on an EU project, a brief guideline for the food preparation operators has been published (Bolton & Maunsell, 2004).

General Principles of Meat Safety Management

Today, a longitudinal-integrated approach to meat safety assurance is widely adopted (Buncic, 2006), with the main responsibility for meat safety resting with producers whose responsibilities include compliance with EC-Hygiene Pack and implementation of GMP/GHP/ HACCP-based systems. Governments have a more advisory, and official control- and audit-orientated role. From the operational perspective, the system comprises several global activities.

Preliminary activities include defining hazard-meat combinations of particular relevance (via epidemiological data, risk profiling) and traceability.

Evaluation of risk management approaches includes identification of available (science) and selection of the "most appropriate" (decision making) control options.

Implementation of control measures includes those "owned" by the producers e.g. Good Manufacturing/Hygiene Practices (GMP/GHP) pre-requisite programmes and Hazard Analysis and Critical Control Points (HACCP) plans; as well as regulatory-mandated procedures and criteria. For example, the most common, generic Critical Control Points (CCPs) where the most important measures are implemented to control the highest meat safety risks include

- On-farm: presently, CCPs are under development as HACCP plans are not yet mandatory for farms.
- In animal feed factories: receipt of various incoming materials (ingredients), batch mixing, and feed heating ("oven").
- In abattoirs: acceptance of animals, skinning, evisceration and chilling in cattle/sheep abattoirs, and some others such as scalding in pig and poultry abattoirs.
- In cutting plants and re-packaging centres: receipt of meat, pre-cut inspection, chill storage and dispatch-transport (if under the operator's control).

All HACCP-based meat safety systems implemented by the industry at various phases of the meat chain are subject to regulatory verification (which often includes microbiological testing for indicator organisms and *Salmonella*) and auditing. In the EU, the new regulation (EC 2073/2005) introduced "Process Hygiene" and "Food Safety" criteria for *Salmonella* on carcasses and in certain meats, but those should be considered together with other aspects of legislation including HACCP-based checks and official controls.

Follow-up and re-evaluation include identifying new problems (monitoring), assessing the effectiveness of implemented controls (surveillance) and reviewing the whole system if the expected results are not achieved.

Finally, from the perspective of safety management for the entire meat chain, it is important to note that the main focus of the system can differ between foodborne pathogens. While some pathogens (e.g. *Salmonella* spp., *Campylobacter* spp., *Y. enterocolitica* and VTEC) are most efficiently controlled by the main interventions applied in primary production combined with optimisation of the slaughter hygiene, the main controls for others (e.g. ubiquitous *L. monocytogene*s) are focused on the post-harvest stage.

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Chapter 2
Fate of *Escherichia coli* O157:H7 in Meat

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Introduction

In the United States, the Center for Disease Control and Prevention (CDC) estimates that the number of foodborne illnesses annually is approximately 76 million cases, resulting in 325,000 hospitalizations and 5,000 deaths. Of those, almost 14 million cases of foodborne illness, 60,854 hospitalizations, and 1,800 deaths are caused by known foodborne pathogens (Mead et al., 1999). The cost of human illness, medical expenses, and productivity losses associated with the six most dominant foodborne pathogenic bacteria has been estimated to be between $2.9 and $6.7 billion dollars per year (Buzby et al., 1996). For decades the meat industry has been the center of some of the most costly outbreaks in world history.

Escherichia coli O157:H7 has been a major concern in the meat industry for decades and has increasing concerns with the development of new processing techniques. *E. coli* O157:H7 has been associated with food since 1982, but *E. coli* O157 is naturally found in the intestinal tract of cattle and in cattle feces (Rodríquez & McLandsborough, 2001). A cascade effect of *E. coli* O157:H7 can be seen during the slaughter and production process. *E. coli* O157:H7 in the feces of cattle can be transferred to the hide. The feces on the hide are transferred to the carcasses during the de-hiding process and from the carcass the knives and saws become a vector to transfer *E. coli* O157:H7 onto other cuts of meat. The contaminated cuts of meat are then ground and added to other animal's cuts of meat. This is a possible cascade of events that can lead to massive amounts of ground products contaminated with *E. coli* O157:H7. The Hazard Analysis and Critical Control Points (HACCP) system and other quality programs have been established to reduce the risk of possible pathogenic contamination during manufacturing process. According to Food Safety and Inspection Service (FSIS) in 2007, *E. coli* O157:H7 was linked to 21 recalls

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of meat products resulting in 90 infected persons with a foodborne illness and 33,358,521 pounds of product lost (Food Safety and Inspection Service, 2008).

How specifically *E. coli* O157 interacts with meat provides a greater understanding of areas within the production process that require an intervention step applied. In this chapter we will explore the different factors that influence "The Fate of *Escherichia coli* O157 in Meat". This topic has been explored in Food Microbiology, Food Science, Biology, and Meat Science with the goal of providing a piece of this complex puzzle to create a safer food supply. Evaluation of patterns of recalls provides more details into where food safety needs to focus, the physiology and conditions of survival of *E. coli* O157 can aid in the development of intervention techniques as well as exploration of vectors that can be used to transfer *E. coli* from objects/instruments or equipment to meat products. Basic knowledge of the physiology of whole muscle cuts can provide insight into the specific locations where pathogens can be transferred. New food safety considerations with new techniques of processing like needle injection and need tenderization must be considered as well as an evaluation of current interventions can detail methods that have been shown to be successful in the reduction of *E. coli* O157:H7 on carcasses and in the meat. All these topics help to clarify the pieces to this massive puzzle.

Escherichia coli O157:H7

E. coli is a gram-negative, facultative anaerobic, non-spore-forming rod, which belongs to the Enterbacteriaceae family. Theodor Escherich first cultured 'Bacterium coli' in 1885 from the feces of a healthy individual. It was renamed *Escherichia coli* in 1919 in a revision of bacteriological nomenclature (Law, 2000). Many benefits have been found from *E. coli* in human medicine, food industry, and the water industry. Some studies suggest that *E. coli* can serve as a benefit to the human body by synthesizing vitamin K and by using competitive inhibition to out compete other bacteria that might enter the intestinal tract.

Differences between strains of *E. coli* lie in the combination of different antigens they possess. There are three types of antigens: the somatic lipopolysaccharide antigen (O), the flagellar antigens (H), and the capsular antigens (K). There are approximately 174 O antigens, 56 H antigens, and 103 antigens that have been identified. There are several stains of *E. coli* that have been isolated. The enteric *E. coli* are divided on the basis of virulence properties into enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), verotoxigenic (VTEC), enterohemorrhagic (EHEC), and enteroaggregative (EaggEC). ETEC can be found in humans, pigs, sheep, goats, cattle, dogs, and horses; EPEC is found in humans, rabbits, dogs, cats, and horses; EIEC and EAggEC are only found in humans; VTEC is found in pigs, cattle, dogs, and cats; while EHEC is found in humans, cattle, and goats and attack porcine strains that colonize the gut in a manner similar to human EPEC strains (Fratamico et al., 2002).

There are several differences between the *E. coli* O157:H7 and other strains of *E. coli*. *E. coli* O157:H7 has a genome size of 5.4 Mb, Uropathogenic *E. coli* 5.2 Mb, and K12 5.2 Mb. *E. coli* O157:H7 and *E. coli* K-12 have many similarities in their genomes. It has been reported that 4.1 Mb are shared commonly on the backbone. Differences lay within the genes that code for the O strain and the K strain. There are 1.34 Mb that code for 1,378 genes in the O strain and 0.53 Mb coding for 528 genes in the K strain. Within the backbone there are 106 islands in the same location that have either O or K islands. K12 has a rough colony type because it has a partial LPS, while O157:H7 has a smooth colony type because it has a capsule and a full LPS. K12 does not have any toxins, adhesion factors, iron transport systems, capsule, or plasmids while O157:H7 does (Riley & Saier, 2007).

Human Health Concerns with *E. coli* O157:H7

E. coli O157:H7 was first described in 1975 in California after it was isolated from a woman with bloody diarrhea, but its identification as an enteropathogen was not until two, nearly simultaneous, U.S. outbreaks during 1982 (Ingham et al., 2006; Wells et al., 1983). It is considered a serious threat to public health in developed countries. In the United States alone, it is the single greatest cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Andreoli et al., 2002). *E. coli* O157:H7 causes the majority and most severe outbreaks of gastrointestinal illnesses related to *E. coli* (Peacock et al., 2001) from infections that range from asymptomatic conditions to mild bloody diarrhea or even severe hemorrhagic colitis. Severity of symptoms usually depends on status of the person infected with the pathogen, with the very young or immunocompromised suffering the most severe episodes.

Infection with *E. coli* O157:H7 can cause a wide variety of outcomes (Food Safety and Inspection Services, 2008; Ingham et al., 2006; Paton & Paton, 1998), with cases being reported worldwide. Bloody diarrhea (or hemorrhagic colitis, HC) caused by *E. coli* O157:H7, where infection of the large intestine occurs, is clinically different from that produced by other gastrointestinal pathogens. Clinical symptoms range from 1 to 8 days, with an average incubation period of 3 days (Peacock et al., 2001). Initially, patients develop abdominal cramps and watery diarrhea, with a varying percentage of these patients' diarrhea resolving without further complications. The cramps can be very severe, with the cecum and ascending colon as the most affected areas that can mimic an acute abdomen inflammation and lead to exploratory laparotomy. Fever is usually absent or mild but occasionally can exceed 102°F (38.9°C). In mild disease without bloody diarrhea, patients have less abdominal cramps, vomiting, and fever and are less likely to develop systemic sequelae, hemolytic uremic syndrome (HUS), or to die (Su & Brandt, 1995). The occurrence of bloody diarrhea can happen as often as 15–30 minutes. Vomiting is also reported in

about 30–50% of cases. Approximately 95% of the cases of HC resolve completely without further complication, however, the remaining 5% develop hemolytic uremic syndrome.

Hemolytic uremic syndrome, a term used for the first time in 1955, is defined as a disorder where kidney failure, hemolytic anemia, and thrombocytopenia (platelet deficiency) develops, usually after 7 days of the onset of diarrhea (Elliott & Robins-Brown, 1993). These symptoms are also accompanied by coagulation defects and variable nervous system signs (Hilborn et al., 1999; Elliott & Robins-Brown, 1993). The pathogen avoids expulsion from the body through its virulence factors causing an attachment that induces the transfer of verotoxin to the mucosa where it is transported by the epithelial cells and absorbed by the gut wall (Hilborn et al., 1999; Law, 2000; Paton & Paton, 1998).

History of *E. coli* O157:H7 in Meat Industry

Meat inspection was practiced in France as early as 1162, in England 1319 and in Germany 1385. In the United States meat inspection has been noted in the 1800s but mandatory inspection did not occur until 1906 with The Meat Inspection Act (Aberle et al., 1975). The government was stimulated by the release of the book *The Jungle* written by Upton Sinclair published in 1904. *The Jungle* reported poor food safety practices observed in the meat industry. The book outlined several areas during slaughter and manufacturing that needed further food safety implementation methods. The Meat Inspection Act of 1906 began the recognition and new regulatory standards that are mandatory to meat industry even today.

In 1981 and 1985 Congress passed several laws that focused on the inspection system focusing on the transmission of disease from animal to humans during consumption (Aberle et al., 1975). The need for wholesome products and the evaluation of live animals prior to slaughter for health concerns which included small butchers and farmers. The poultry industry soon followed with inspection regulations in 1957 with the Poultry Products Inspection Act. In 1967 and 1968 congress passed the Wholesome Meat Act and the Wholesome Poultry Products Act to ensure that processing plants would be held liable for the products being produced in their facilities (Aberle et al., 1975).

Many other laws were granted in years to follow, but education and training of employees were lacking in government documents. In 1986 the Processed Products Inspection Improvement Act provided a resource to the meat inspectors on how to allocate training and increase the overall effectiveness of product inspection. These measures were evaluated in the early 1990s when there was several highly publicized food poisoning with *E. coli* O157:H7 and meat products. In 1996 the USDA mandated the implementation and use of the HACCP in meat and poultry plants to help with food safety and aid as a prevention method. HACCP is designed to identify safety hazards that can by controlled

and monitored during the food production process. HACCP was first introduced by Pillsbury Company in 1959 to assure that food produced for NASA astronauts had a safe food supply during space travel (Aberle et al., 1975). The concept of prevention instead of reaction of hazards in the food resulted in a renewal of training and education to meat suppliers and producers. Sanitation practices, pre- and post-operational procedures, flow diagrams of all products produced and methods of slaughters as well as all forms used to monitor critical control points are documented in a record keeping section. Critical control points are steps within the production process that can reduce or eliminate the potential for a hazard (chemical, biological, or physical) to enter into the food product (Aberle et al., 1975).

HACCP and other programs (i.e., ServSafe) along with the USDA continual renewal of rules for the meat industry have re-ensured the consumers that the United States provides one of the safest supplies of food. FSIS along with universities throughout the world have collaborated to keep the latest information of pathogens and meat products current. *E. coli* O157:H7 has been a reoccurring problem in the meat industry.

The presence of *E. coli* O157:H7 in feedlots along with the presence in cattle feces has provided a challenge for cattle producers and packing plants. It is inevitable that cross-contamination will occur in these conditions. From 1998 until May 31, 2008, the pattern of outbreaks with *E. coli* O157:H7 and meat products reflects both changes in meat processing and increase in sampling in the processing plants. Figure 2.1 displays the pattern of recalls associated with *E. coli* O157:H7 and meat products over the past 10 years. From 2000 to 2002 there was a pattern of higher amount of recalls in meat products with *E. coli* O157:H7 in the United States. During this period a higher amount of smaller recalls with grocery food chains were reported. In many cases of these recalls less than 200 lbs of product was included in the grocery recall.

In October 2002, FSIS ordered all beef plants to re-examine their food safety plans, based on evidence that *E. coli* O157:H7 is a hazard reasonably likely to occur and to implement interventions to prevent it (Food Safety and Inspection Service, 2002). Scientifically trained FSIS personnel then began to systematically assess those food safety plans for scientific validity and to compare what was written to what was taking place in daily operations. A majority of the meat processing plants have made major changes to their operations based on the directive, including the installation and validation of new technologies specifically designed to combat *E. coli* O157:H7. Many plants have also increased their testing for *E. coli* O157:H7 in order to verify their food safety systems (Food Safety and Inspection Services, 2002).

Beginning in January of 2003 the Beef Industry Food Safety Council (http://www.bifsco.org/BestPractices.aspx) took representatives from every sector of the beef industry and diligently working together on unified best practice documents that will serve as a blueprint for making beef an even safer product. These documents are available to animal producers, slaughter facilities, and retail stores on the best practices to use to ensure a safer meat product. These

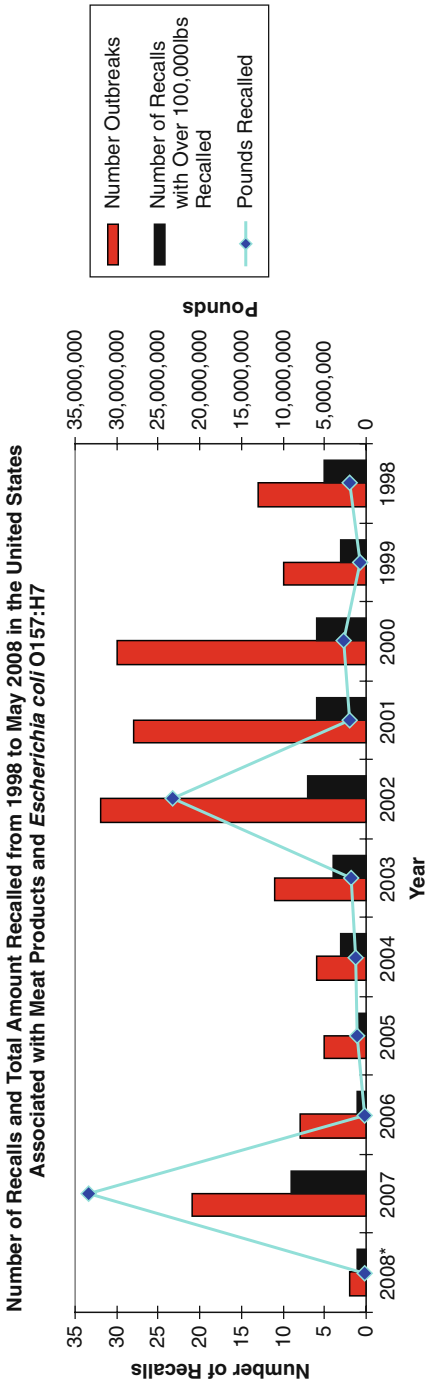

Fig. 2.1 Number of recalls and total amount recalled from 1998 to May 2008 in the United States associated with meat products and *Escherichia coli* O157:H7 according to the Food Safety and Inspection Services

documents are regularly updated with the newest research findings (Beef Industry Food Safety Council, 2008).

As a result of these actions, in 2003 the U.S. Department of Agriculture's Food Safety and Inspection Service released data showing a drop in the number of *E. coli* O157:H7 positive samples in ground beef collected compared with previous years (Food Safety and Inspection Services, 2002). A noticeable reduce was observed in both number of recalls and the number of recalls resulting in more than 100,000 lbs requiring recalling status was observed (Food Safety and Inspection Services, 2008).

In 2007 there was another spike in recalls in the United States. In October 2007, the second largest meat recall of 21.7 million pounds of meat was announced. Forty cases of *E. coli* O157:H7 infections have been identified with PFGE patterns that match at least one of the patterns of *E. coli* strains found in Topp's brand frozen ground beef patties. The ill persons from this outbreak resided in eight states [Connecticut (2), Florida (1), Indiana (1), Maine (1), New Jersey (9), New York (13), Ohio (1), and Pennsylvania (12)]. Two patients developed a type of kidney failure called hemolytic uremic syndrome (HUS) with no deaths reported (Food Safety and Inspection Services, 2007a). On the heels of this massive recall, Cargill Meat Solutions Corporation, a Wyalusing, Pennsylvania, firm, was voluntarily recalling approximately 1,084,384 pounds of ground beef products because they may be contaminated with *E. coli* O157:H7, the U.S. Department of Agriculture's Food Safety and Inspection Service announced November 3, 2007. This recall affected over 30 different ground beef products distributed throughout the United States (Food Safety and Inspection Services, 2007b).

Immediately the FSIS responded to these high volume recalls investigating the food safety practices observed in these plants. Six days after this recall was issued, after 67 years of business Topps Meat Company was closed. This conclusion was indefinite after such a large recall. Hudson Foods Co. closed its plant in Columbus, Neb., after it agreed in 1997 to destroy 25 million pounds of hamburger in the largest U.S. meat recall after E. coli was found in the ground beef. The plant later reopened with new owners (Associated Press, 2007). The Food Safety and Inspection Services (FSIS) continues to monitor recalls and conducts research with many universities to ensure that the newest technologies and intervention tactics are used.

Sources of *E. coli* O157:H7 Cross-Contamination

There are many vectors that can be used to transfer *E. coli* O157:H7 on and/or into meat products. The feces of the animal can be transferred on the hides and carcass, the equipment can be contaminated, personnel might not use proper hygienic practices, airborne contamination, and rodents, insects, and other animals are all potential sources.

Depending on the sample size of hides and carcasses and the location of the study a range of between 1 and 40% prevalence of *E. coli* O157:H7 has been reported (Arthur et al., 2002; Bonardi et al., 2001). In a study with 355 beef cattle in the United States the scientist reported a 17% prevalence of positive samples for *E. coli* O157:H7 and a strong correlation between the fecal and hide prevalence with the carcass contamination (Elder et al., 2000). Several steps within the de-hiding process have been identified as causing the most cross-contamination of fecal matter. Hygienic practices in a slaughter facility can influence the presence of *E. coli* O157:H7 on carcasses and in the environment. Heuvelink, Roessink, Bosboom, and Boer (2001) visited several slaughter facilities and sampled the brisket, flank, and back surfaces and reported that 39% of the 27 slaughter plants visited had inadequate hygiene practices, resulting in 50% of the carcasses having visible contamination of feces that must be cut away. Arthur et al. (2002) reported that 56% of the hide samples in three abattoirs were positive for *E. coli* O157 with 41% of these being below 60 MPN/100 cm^2. In this same study 14.7% of the carcasses were positive for *E. coli* O157 with 83% of these below 1.3 MPN/cm^2. In this study the researches concluded that the rump region on the carcass was identified as having the most contaminated with fecal organisms than the other sites in one study and was linked to the skinning process and the presence of more fecal and dirt matter prior to slaughter (Bell, 1997; Gill et al., 1996). Besides the rump site, the hindquarter and flank were identified in three beef slaughtering facilities in a Canadian study (Gill et al., 1998).

Some researches believe that during transporting cattle to the slaughter facility that cattle *E. coli* O157:H7 might be shred and aid as a method for cross-contamination. Minhan et al. evaluated the influence of lairage and transportation in fecal shedding of *E. coli* O157 in cattle. No increase in the prevalence of *E. coli* O157 from farm to dressed carcass was observed. None of the 168 samples were positive for the dressed carcass. This study demonstrated that even positive cohorts of cattle may be slaughtered and processed to produce clean carcasses when hygienic practices are followed (Minihan et al., 2003). Madden et al. found similar results with cattle harvested in Northern Ireland with no positive *E. coli* O157 samples on 780 carcasses (Madden et al., 2001).

Equipment is also another vector for transferring bacteria from surface to meat products. Pathogenic bacteria transfer rates from contact surfaces to food items can be influenced by many factors. Rodriguez and McLandsborough (2001) found that the transfer rate of *Listeria monocytogenes* onto bologna was lowest with less pressure and shorter time on stainless steel surfaces. Oliveira et al. (2006) found that the ability of *Salmonella* to adhere to polyethylene and polypropylene was dependent on strain. *Salmonella* Typhimurium and *Campylobacter jejuni* have also been shown to have the ability to transfer from stainless steel to romaine lettuce after 10 seconds (Moore et al., 2003). Dawson, Han, Cox, Black, and Simmons (2007) also found that *S.* Typhimurium can be transferred almost immediately on contact and can survive up to 4 weeks on a dry tile surface

while maintaining high enough populations to transfer to foods. Ingham et al. (2006) reported that *Streptococcus pyogenes* adhered almost immediately to various plastic, ceramic, and stainless steel utensils and were present at similar inoculated levels for at least 2 hours. Although these studies are not of *E. coli* O157:H7 they do provided evidence that equipment can be a source of cross-contamination due to attachment.

Personnel can also be a source of transfer of *E. coli* O157:H7 to meat products. If personnel do not wash their hands properly or do not change gloves often, then *E. coli* O157:H7 can be transferred from restrooms or from other uncooked meat products. Food safety training can aid in the reduction of potential occurrences with cross-contamination. Many studies have been conducted to determine what influences the retention of food safety training. In 2002 food handlers were given a survey to evaluate their beliefs and it was determined the best way to train. Sixty-three percent of the 127 participants admit to sometimes not carrying out food safety behavior because of lack of time, lack of staff, and lack of resources. It is recommended that risk-based approach and demonstrations can change the behavior of the food handlers (Clayton et al., 2002). In Florida comparison between outbreaks prior to training (1997–2000) and after training (2001–2003) determined that insufficient time or temperature during cooking, cross-contamination, bare-hand contact, insufficient cold and hot holding times were still the major causes of foodborne outbreaks before and after training, while the number of cases reduced from 5,671 prior to 3,568 after training. There conclusion is that training does help with food safety but knowing trends in contributing factors can help to determine areas of focus needed for food safety training (Hammond et al., 2005).

Food industry has the constant challenge of controlling rodents, insects, and other animals out of their plants and storage units. Most plants have adopted multiple hurdles and procedures to minimize entry of such animals. An active and aggressive rodent control plan is necessary to maintain continual control. Along with rodent control, airborne transfer is a potential source of contamination in the plant. The ventilation system needs to be maintained and included in the sanitation plan daily. Bird droppings and animals can live in the ventilation system and result in animal feces and parts to be sprayed during the ventilation of the plant. Also during the cleaning process, *E. coli* O157:H7 can be aerosolized and remain in the air for some time depending on the droplet size. Fans and other air circulation systems must be used with caution in environments where raw products can interact with cooled or further processed products.

There are many sources where contamination can occur such as airborne transfer, rodent contamination, hide/carcass transfer of pathogen, and personnel poor habits. Education to personnel and proper sanitation standard operating procedures (SOPs) can aid in the reduction in the transfer of *E. coli* O157:H7 and other pathogens throughout the plant and into the product.

Survival of *E. coli* O157:H7

Studies have focused specifically in the growth, survival, and inactivation characteristics of this pathogen (Bell, 1998; Juneja & Marmer, 1999; McClure & Halls, 2000). Studies have been performed to understand the behavior of *E. coli* O157:H7 in different substrates and foods for varying periods of time, as well as the effect that different intrinsic and extrinsic factors such as hot and cold temperatures, pH, organic acids, water activity (Aw), salt, control of reduction–oxidation potential (RO), fat content, irradiation, and preservatives will have on this specific pathogen. *E. coli* O157:H7 can be controlled by proper cooking of the food product to a specific temperature and time.

E. coli O157:H7 is a cause for concern especially if present in foods that do not go through a treatment process to eliminate the pathogen, or that could be contaminated after such process and before packaging as in the case of ready-to-eat (RTE) products. After the outbreak in 1993 (Anonymous, 1993; Bell et al., 1994), the USDA considered *E. coli* O157:H7 an adulterant if present in ground beef, setting for the first time in the United States' history a zero tolerance policy for the presence of a microorganism in raw meat product (Heuvelink et al., 2001; Hollingsworth & Kaplan, 1997; Todd, 2004; Tuttle et al., 1999). Some examples of the foods involved with *E. coli* O157:H7 outbreaks include ground beef (Anonymous, 1993; Bell et al., 1994; Brandt et al., 1994) and meat products (Anonymous, 2007; Jay et al., 2004; Laine et al., 2005); apple juice (Anonymous, 1996; Besser et al., 1993; Cody et al., 1999); radish (Michino et al., 1999); raw sprouts (Anonymous, 1997); lettuce (Ackers et al., 1998; Hilbourn et al., 1999); and other types of fresh vegetables including baggy salads (Ackers et al., 1998; Anonymous, 2007; Sivapalasingam et al., 2004), as confirmed by the recent spinach outbreak in California.

Microorganisms are not killed instantly when exposed to a lethal agent, but rather, the population decreases exponentially. The D value or "decimal reduction time" is used in food microbiology to describe at any given temperature the time required in minutes to reduce 90% (or 1 log) of a specific microbial population in a specific food, and it is affected by factors such as pH, water activity (Aw), content of preservatives, product composition, and the size of the microbial population, among others. Studies have revealed that cooking ground beef with 17–20% fat at 57.2°C and 62.8°C have D values of 4.5 and 0.40, respectively. Cooking hamburgers to an internal temperature of 71.1°C (160°F) for 15 seconds is required to assure adequate cooking and prevent outbreaks (Doyle et al., 1997; Pflug & Holcomb, 1977; Stumbo, 1973; Wojciechowski et al., 1976).

Pasteurization is also an accepted heating method to destroy this pathogen in milk, fruit juices, and ciders. Treatment of milk for 15 seconds at 71.7°C (161°F) allows a 5-log reduction of *E. coli* O157:H7 and the same reduction is achieved in apple cider when it is pasteurized at 68.1 for 14 seconds (Al-taher & Knutson, 2004; Lawrie, 1998). Other studies have shown recovery of *E. coli* O157:H7 in

artificially inoculated foods after frozen storage. In one study, *E. coli* O157:H7 was recovered from inoculated strawberries, radishes, and cabbage after 2 and 4 weeks of storage at –20°C (Hara-Kudo et al., 2000).

Ground beef used in the manufacturing of hamburger patties is often produced in a central location and distributed under frozen conditions to fast food restaurants in different locations. In the 1993 *E. coli* O157:H7 multistate outbreak involving undercooked hamburgers, contaminated frozen patties produced by a single plant in California were involved with illness 6 weeks after the production date (Bell et al., 1994; Tuttle et al., 1999). Studies performed after that outbreak in inoculated ground beef patties (20% fat) revealed that *E. coli* O157:H7 can survive for up to 4 weeks after storage at –2°C with a 1.5 log reduction in the population. Storage of ground beef at –20°C for 12 months established recovery of the pathogen with an approximate reduction of 1.0 log (Ansay et al., 1999), demonstrating the ability of *E. coli* O157:H7 to survive in hamburgers for long periods of time at frozen temperatures with little decline in numbers of viable cells.

As seen in the examples above, *E. coli* O157:H7 displays a unique ability to survive in a wide variety of products subjected to different process conditions for long periods of time, allowing the foods to serve as vehicles in the transmission of infections.

Physiology of Whole and Ground Meat Products

The physiology of the meat product influences the likelihood of pathogens to be able to adhere and survive over time on the product. Whole muscle cuts have different areas of concerns with *E. coli* O157:H7 then further processed meat products. With intact whole muscle cuts, the interior of the cuts is sterile to vegetative pathogens. Internalization of any pathogenic microorganisms can only occur when the external surface is penetrated exposing the interior by the destruction of the myofibrillar structure of the meat. Meat tenderization methods such as brine injection and basic needle tenderization can place pathogens from the surface or from a contaminated needle into the interior of the whole muscle. The presence of water can also influence the transfer of pathogens internally.

The ability of meat to retain inherent or added water affects such eating attributes as toughness, juiciness, appearance, and the firmness of the bit (Lonergan, 2005). Water is held either inside the muscle cells or in the extracellular space. Largest amount of water is in the myofibrils and between the myofibrils. About 10% of the total water is held in the "I" band. There are many factors that influence the water holding capacity of a meat product. These include pH, protein structure alterations, alterations in the structural components, development of rigor mortis, and addition of substances. The absorption of contaminated water can place *E. coli* O157:H7 into the interior of the meat (Lonergan, 2005).

Also when a product is ground or sliced pathogens can be spread through the meat (Lonergan, 2005). Ground products have a greater probability of exposure to *E. coli* O157:H7 than intact product because ground products have more exposure to equipment and personnel handling. Ground products must be cut up, which is mostly done manually, and then ground in a machine with meat from other animals. Equipment, tables and personnel add to the increased exposure of *E. coli* O157:H7. Ground products are also sold in a raw state that requires consumers to properly cook the product to reduce the chances of illness. The lethality step is placed into the hands of consumers who rarely use thermometers during the cooking process. If this process is performed properly then raw products will be generally free from vegetative pathogens and most spores (survival of spores depends on the specific microorganism that produces it) (Buzby et al., 1996).

Enhanced and Mechanically Tenderized Meat Concerns

Sensory and quality attributes of tenderized meat have been studied extensively by many authors before; however, the microbiological aspects of this process have not received much attention until very recently. It is generally accepted that bacteria associated with meat are derived from the ingesta, the environment and the instruments used in the fabrication of the carcass, occurring only in the surface of the meat (Co, 1979). The internal muscles and deep tissues of the carcass are sterile unless they are subjected to a considerable breakdown of the connective tissue structure and muscle fibers. Similarly, during the process of carcass fabrication, Mechanical tenderization processes (such as blade tenderization, brineinjection or marination) can introduces bacteria into the deep tissues of the subprimals (Gill & Penney, 1979; Gill et al., 2005a; Gill et al., 2005b; Sporing, 1999), which can become a problem if the meat is undercooked.

In an *E. coli* O157:H7 risk assessment for blade-tenderized beef conducted at Kansas State University, beef top sirloin subprimals were inoculated with high levels of the pathogen (106 cfu/cm^2) and subjected to one pass through a needle tenderization unit (Phebus et al., 2000). After evaluation of core samples, the needle tenderization process resulted in about 3.0 logs of the pathogen being translocated into the deep tissues (6 cm from the surface). Samples inoculated at low levels also resulted in a similar trend, with approximately 1.8 logs of the pathogen being transferred into the center of the meat cut. When determining adequate cooking temperatures for the steaks using an oven, the authors also reported that internal temperatures of 140°F and higher were needed to eliminate *E. coli* O157:H7 by broiling.

In another study conducted by Gill and others (Gill et al., 2005), the microbiological conditions of the surface and deep tissues of beef mechanically tenderized at a packing plant were determined. The authors reported that the

tenderizing process did not significantly alter the numbers of bacteria (aerobes, coliforms, and *E. coli*) on the surfaces of strip loins and that none of them were recovered from the deep tissues of treated cuts. When these results are compared to those obtained by Gill and others (Gill & McGinnis, 2004), the results of the packing plant study revealed that the surface counts at retail stores were $\geq$2.0 log10 units more than those obtained in the plant. The authors suggested that not only storage was a factor on the high surface numbers obtained at retail stores, but that the cleanliness of the tenderizing equipment at the packing plant was a major aspect affecting the numbers of bacteria recovered from deep tissues. Other studies have also confirmed that the numbers of bacteria recovered from deep tissues of needle tenderized meat are significantly affected by the number of bacteria in the surface and the penetration depth but not by the number of "incising events" (passes) to which the meat is subjected (Gill et al., 2005a).

As well as with needle tenderization, injection of meats can pose the risk of translocation microbial flora and pathogens that are in the surface of the meat into sterile deep tissues of the cut. Some studies have tested the survival of different pathogens in the brine, a solution that is usually re-circulated and that if contaminated can subsequently inoculate additional cuts; however, just a few studies have focused on the surface-deep tissues translocation levels that can occur while enhancing meat products. Introduction of pathogenic microorganisms into the deep tissues of meat can result in a shorter shelf life and an increase in the risk of foodborne illness (Johnston, 1978).

In a study conducted in Canada, the brine used to pump moisture-enhanced pork was microbiologically analyzed for up to 2.5 hours after recirculation (Greer et al., 2004). The authors reported significant increases in the numbers of bacteria obtained from the brine after 1.75 hours of recirculation. After 2.5 hours of recirculation, the reported log CFU/ml counts were 4.50 (total plate count), 2.99 (lactic acid bacteria), 3.95 (*pseudomonas*), 2.79 (*Brochothrix thermosphacta*), and 3.01 (enterics); indicating that these solutions can harbor significant numbers of spoilage bacteria and can be distributed easily in the meat. In a recent study, the impact of a commercial injection process in the microbial flora of pork loins was studied. Aerobic bacteria recovered from re-circulated brine were >3.5 $\log_{10}$ units more than those obtained from the preparation tank after 30 minutes of processing (Gill et al., 2005c).

Similarly, other authors have tested the survival of pathogens in the brine and its effects on enhanced products. In one study, brine used to enhance eye of round primal cuts was inoculated with cultures of *L. innocua*, and portions of meat and brine analyzed after injection (Gill et al., 2008). The authors reported that the levels of this pathogen in the meat were about 0.72 $\log_{10}$ units less than those obtained in the brine. The authors also suggested that factors such as pumping pressure and number of strokes per minute can also affect the amount of brine (and therefore, pathogens) retained by the meat. Additionally, the authors suggested that if a meat product is subjected to both needle

tenderization and injection with brine, the enhancement process must be performed prior to the tenderization process to reduce the levels of possible contamination retained by the meat.

In a study conducted at Colorado State University, decontamination methods for *E. coli* O157:H7 were tested on beef subprimal cuts intended for moisture enhancement. Inoculated meat cuts were treated with hot water, lactic acid, and activated lactoferrin among other interventions and then injected with a brine solution containing 0.5% sodium chloride, 0.25% sodium tripolyphosphate, and 2.5% sodium lactate (Heller et al., 2007). The authors reported that treatment of the meat cuts with the interventions resulted in 0.9–1.1 $\log_{10}$ cfu/100 cm^2 reduction (a significant reduction when compared to the control samples); however, no significant differences among treatments were found. When internal swab surfaces were analyzed, the process resulted in $<1.05\%$ cfu/cm^2 of surface pathogen transferred into the meat.

Intervention Strategies

Microbial contamination of meat with pathogens such as *E. coli* O157:H7 and *Salmonella* is a public health concern due to the outbreaks of foodborne illness commonly associated with the consumption of these products. The need to prevent these unfortunate incidents has prompted the incorporation of different types of control measures in the processing facilities in order to reduce and eliminate these pathogens from the food products and to prevent them from entering the food supply. Contamination of the carcasses can occur in different steps during the slaughter process, especially during de-hiding and evisceration of the animal.

As part of the adoption of the HACCP system, all beef processors and plants need to develop a plan that identifies the hazards that are associated with their respective process and the control measures that can be implemented in each step to reduce their likelihood in the food product. In the U.S. meat industry some of these control measures are known as interventions, proven procedures that significantly reduce microbial contamination from the meat surfaces, with many of them being used in sequence as part of a multiple hurdle approach. These control measures can be categorized into (a) physical (hot water spray, steam pasteurization, steam-vacuuming, water wash cabinet, and knife trimming); (b) chemical (organic acids, polyphosphates, chlorine, acidified sodium chlorite, ozone, peroxyacetic acid, nisin, and lactoferrin); (c) emerging technologies (hydrostatic pressure, irradiation, pulsed electric fields, and microwaves) (Samelis, 2005); and (d) biological (lactic acid bacteria and bacteriophages). The use of the previous interventions and their effectiveness on beef hides (Acuff, 2005; Koohmaraie et al., 2005), carcasses (Keeton & Eddy, 2004) beef trim/variety meats, and ground beef (Snijders et al., 1985; Koohmaraie et al., 2005) have been reported by previous authors, and they are often used in

addition to other procedures, such as inspection of the carcass and knife trimming of any visible feces, ingesta, hair, lesions, or bruises (USDA-FSIS, 1996). The effectiveness of the interventions and the levels of bacterial reduction that are obtained vary according to the testing methodologies that are used and the type of meat surface that has been tested, often leading to diverse results. Additionally, the concentration of the acid and its pH also determines the effectiveness of the compound against bacterial loads (Snijders et al., 1985). Examples of the effectiveness of different spray interventions in beef carcasses and meat products are summarized on Table 2.1. It is worth noting that even though interventions can reduce the risk of pathogens to be transferred to meat surfaces and their final products, they do not provide 100% assurance of safety. In addition, the se of interventions should not be viewed as a way to "clean" unwholesome products and in no case they can be a substitute for strict hygienic manufacturing practices and good cleaning and sanitation procedures in the processing facility.

In different studies conducted at Texas Tech University, the effectiveness of lactic acid producing bacteria ($\sim 10^7$ cfu/ml), acidified sodium chlorite (1,000 ppm), and lactic acid (3%) as intervention strategies to control *E. coli* O157:H7 and *Salmonella* Typhimurium DT 104 in non-intact beef products have been evaluated and proved effective against these pathogens (Echeverry, 2007). In one of the studies, inoculated boneless beef strip loins sprayed individually with the interventions and subjected to mechanical tenderization after 14 or 21 days of aging presented significantly lower *E. coli* O157:H7 counts in the internal muscle (between 1.2 and 2.0 logs) by the application of lactic acid and lactic acid bacteria (Echeverry et al., 2008a). In an additional study, inoculated strip loins sprayed with the interventions after 14 days of aging and followed by injection with a brine solution also presented lower internal *E. coli* O157:H7 counts (up to 2.0 logs) after the application of lactic acid bacteria and acidified sodium chlorite (Echeverry et al., 2008b).

Conclusion

E. coli is the most researched bacteria in the microbiological field. *E. coli* O157:H7 has been the cause of multiple outbreaks of food borne illness in the United States for more than a decade. The financial impact of producers and health concerns has caused an increase in research and acknowledgement of need. A growing population of people creates a need for food production that provides quality and safe products manufactured efficiently.

There are several areas within the meat industry that require further research to help in the continual goal of providing the safest product to consumers. Continual monitoring of *E. coli* and meat products, updating information about the physiology of *E. coli* O157:H7 and mutational changes.

Table 2.1 Selected studies on the effectiveness of different interventions sprays and dipping solutions used in the US meat Industry to control and eliminate different microbial pathogens in beef tissues

Interventions	Concentration (temperature)	Microorganism	Log_{10} reduction	Tissue/product	References
Hot Water Wash	N.A[2](76–80°C)	Aerobic Plate Counts	4.3 CFU/cm^2	Beef tissue	Anderson, Marshall, Stringer, and Naumann (1979)
Trimming + HWW[1]	N.A (35°C)	*E. coli* O157:H7	4.7 CFU/cm^2	Beef carcass	Phebus et al. (1997)
Hot Water Spray	N.A (95°C)	*E. coli* O157:H7	3.7 CFU/cm^2	Beef Carcass	Castillo, Lucia, Goodson, Savell, and Acuff (1998)
		Salmonella Typhimurium	3.8 CFU/cm^2		
		Aerobic Plate Counts	2.9 CFU/cm^2		
		Total Coliforms	3.3 CFU/cm^2		
Water Wash	N.A (35°C)	*E. coli* O157:H7	3.3 CFU/cm^2	Outside beef rounds	Castillo et al. (2001)
		Salmonella Typhimurium	3.4 CFU/cm^2		
Water Wash + Lactic Acid	2.0% (55°C)	*E. coli* O157:H7	5.3 CFU/cm^2	Outside beef rounds	Castillo et al. (2001)
		Salmonella Typhimurium	5.4 CFU/cm^2		
Lactic Acid	2.0% (55C)	Aerobic Plate Counts	>0.9 CFU/cm^2	Beef Short Plate Pieces	Rose et al. (2004)
		Total Plate Counts	>1.0 CFU/cm^2		
	2.5% (55°C)	Aerobic Plate Counts	>1.0 CFU/cm^2	Beef Short Plate Pieces	
		Total Plate Counts	0.8 CFU/cm^2		
Lactic Acid	2.0% (N.A)	Total Mesophylic Bacteria	~1.0 CFU/cm^2	Beef carcasses	Dormedy, Brashears, Cutter, and Burson (2000)
		Total Psychrotrophic Bacteria	~1.0 CFU/cm^2		
		Total Coliforms	>0.7 CFU/cm^2		
		Total *E. coli*	>0.8 CFU/cm^2		
Lactic Acid	2.0% (N.A)	*Salmonella* spp.	>0.5 CFU/cm^2	Beef Trim	Harris, Brashears Brooks, and Miller (2005)
			0.4 CFU/g	Ground Beef	

Table 2.1 (continued)

Interventions	Concentration (temperature)	Microorganism	Log_{10} reduction	Tissue/product	References
Lactic Acid	2.0% (N.A)	*E. coli* O157:H7	> 2.0 CFU/g	Beef Trim	Harris, Miller, Loneragan, and Brashears (2006)
		Salmonella Typhimurium	> 1.0 CFU/g		
Acetic Acid	4.0% (N.A)	*E. coli* O157:H7	> 2.0 CFU/g	Beef Trim	Harris et al. (2006)
		Salmonella Typhimurium	> 1.2 CFU/g		
Acidified Sodium Chlorite	1,000 ppm (N.A)	*Salmonella* spp.	0.5 CFU/cm^2	Beef Trim	Harris et al. (2005)
			0.6 CFU/g	Ground Beef	
Acidified Sodium Chlorite	1,200 ppm (NA)	*E. coli* O157:H7	>1.5 CFU/g	Beef Trim	Harris et al. (2006)
		Salmonella Typhimurium	>1.1 CFU/g		
Acidified Sodium Chlorite	1000 ppm (N.A)	*E. coli* O157:H7	0.6–0.8 CFU/cm^2	Beef Briskets	Hajmeer, Marsden, Fung, and Kemp (2004)
		Staphylococcus aureus	0.8 CFU/cm^2		
Acidified Sodium Chlorite	1,200 ppm (22.4–24.7°C)	*E. coli* O157:H7	3.8–4.5 CFU/cm^2	Beef Carcasses	Castillo, Lucia, Kemp, and Acuff (1999)
		Salmonella Typhimurium	3.9–4.6 CFU/cm^2		

[1] Interventions: HWW (Hot Water Wash)

[2] N.A: Not applicable/not available

Updating food safety concerns with new meat products (i.e., meat tenderized and *E. coli* internalization) and development and evaluation of intervention strategies are some areas that need continual research and monitoring. The Food Safety and Inspection continues monitoring recalls and conducts research with various universities to ensure that the newest technology and intervention tactics are used.

The fate of *E. coli* O157 in meat includes many sectors of the food science/meat science discipline which provide pieces to a complex puzzle of factors. Evaluation of recall patterns, the physiology/survivability of *E. coli* O157, identification of vectors of transfer of *E. coli* O157, the physiology of meat products, non-intact meat products concerns (enhancement, mechanical tenderization), and evaluation of current intervention methods all aid in the exploration of the fate of *E. coli* O157:H7 in meat.

Food safety will continue to be the number one concern of the United States government and through the collaboration of many disciplines in food science the goal for the safest food supply in the world will continue to be reached.

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Chapter 3
Insights into Fresh Meat Spoilage

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Development of Spoilage Microbiota

The conditions under which the animals are reared and slaughtered determine the level, extent and type of contamination. Possible sources of contamination include the abiotic environment in contact with the animal (air, soil, water, feeds), the animal itself (hides, intestinal tract, faeces) and the processing equipment including utensils and humans. Contamination may also vary according to specific characteristics of each animal, its geographic origin as well as the season of the year.

The micro-organisms that usually dominate the initial microbiota of fresh carcasses are Gram-negative rods (mainly pseudomonads) and micrococci (mainly *Kocuria* spp. and *Staphylococcus* spp.). Furthermore, Gram-negative bacteria such as *Acinetobacter* spp., *Alcaligenes* spp., *Moraxella* spp. and Enterobacteriaceae, and Gram-positive species including spore-forming bacteria, lactic acid-producing bacteria and *Brochothrix thermosphacta*, as well as yeasts and moulds, may also be present in small numbers.

Growth and development of the spoilage microbiota of fresh meat is governed, as in the case of all foodstuffs, by

(i) intrinsic parameters of the meat, such as pH and buffering capacity, water activity, Eh and poising capacity, presence of antimicrobial compounds and nutrient composition,
(ii) type and extent of processing,
(iii) extrinsic factors such as temperature, relative humidity and the composition of the gaseous atmosphere,

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(iv) implicit factors, including antagonism and synergism and
(v) interactive effects of the above mentioned factors, other than that expected from their individual action.

All these ecological determinants influence the establishment of a particular microbial association and determine the rate of attainment of a maximum population known as the 'ephemeral (specific) spoilage micro-organisms' (E(S)SO), i.e. those that are able to adopt various ecological strategies. The latter are developed as a consequence of environmental determinants and allow the micro-organisms to proliferate and eventually dominate an environmental niche. Thus, in raw meat of low and high pH that is stored aerobically at cold temperatures, *Pseudomonas* spp. and *Shewanella putrefaciens* are considered to be the main spoilage bacteria (Garcia-Lopez, Prieto, & Otero, 1998). On the other hand, *B. thermosphacta* and lactic acid bacteria dominate during storage of meat under vacuum or other modified atmospheres (Stanbridge & Davies, 1998).

Apart from the imposed environmental conditions, microbial interactions play an equally important but still not fully exploited role in the development of the microbial association (Nychas, Drosinos, & Board, 1998; Tsigarida, Boziaris, & Nychas, 2003). Study of these interactions is important in understanding spoilage. Gram and Dalgaard (2002) reported that *Pseudomonas* spp. could inhibit the growth of *S. putrefaciens* due to its ability to produce siderophores. Moreover, competition for nutrients (e.g. glucose), metabiosis (production of a favourable environment) and cell-to-cell communication (quorum sensing) could also affect the physiological attributes of the organisms under the imposed ecological determinants (Drosinos & Board, 1994; Drosinos & Nychas, 1997; Lambropoulou, Drosinos, & Nychas, 1996). Indeed, it has been reported that the chemical changes occurring in naturally contaminated fish and meat were found to be significantly different from those on sterile muscle tissue when it was individually inoculated with the ephemeral spoilage micro-organisms (Koutsoumanis & Nychas, 1999; Tsigarida & Nychas, 2001). Studies in co-culture model systems were found to be helpful in simplifying the natural food ecosystem and providing an insight into possible interactive behaviours during the development of potential ephemeral spoilage micro-organisms. Furthermore, they may prove themselves useful in identifying metabolites that may be further used as a unique chemical spoilage index (Tsigarida et al., 2003).

The contribution of nutrients to either antagonistic or synergistic interactions has also been the case of intensive study. The principal carbon source, namely glucose, has been found to be metabolized more rapidly by the obligate aerobic strains of pseudomonads, in comparison to the facultative anaerobic strains of *B. thermosphacta* and oxidative strains of *S. Putrefaciens* (Tsigarida et al., 2003). Although their growth rate was not affected by co-culturing with either *Shewanella* spp. or *B. thermosphacta*, an acceleration of glucose consumption was evident. It was concluded the pseudomonads can play a syntrophic role for *Brochothrix* spp. This observation is of great importance since *B. thermosphacta* has a much greater spoilage potential than lactobacilli and

can be important in both the aerobic and anaerobic spoilage of muscle foods. On the other hand, a typical antagonistic interaction that affects the selection of spoilage flora is evident in the case of pseudomonads and *S. putrefaciens*. It is well established in the literature that the inhibitory effect of the former bacterium over the latter is attributed to the ability of *Pseudomonas* spp. to produce siderophores (Gram & Dalgaard, 2002). However, in this case, competition for glucose seems also to play a critical role in *Pseudomonas* spp. dominance. Another example of the interactive properties of Gram-negative spoilage microbiota is their ability to produce chemical communication signals such as acylated homoserine lactones (AHLs). It has been shown that these AHL compounds can be found in wide range of foods including fish, meat and vegetable products (Smith, Fratamico, & Novak, 2004) in concentration proportional to the growth of Gram-negative bacteria. The role of AHLs in muscle food spoilage is currently unknown, but several phenotypes (pectinolytic, lipolytic, proteolytic and chitinolytic activities) potentially involved in spoilage of different foods have been linked to AHL regulation in several bacteria (Gram & Dalgaard, 2002). Elucidation of their role in muscle food spoilage will be an important area for future research.

All the physicochemical changes that occur in fresh meat take place in its aqueous phase. There are three classes of substances that are utilized by spoilage microbiota:

(i) compounds involved in the glycolytic pathway (e.g. glucose, glucose-6-P)
(ii) metabolic products (e.g. lactate)
(iii) nitrogen energy sources (e.g. amino acids, proteins)

The low-molecular-weight compounds, especially carbohydrates and their intermediate catabolic products, are preferentially utilized by the meat microbiota as energy source. Depletion of these substrates will inevitably lead to an amino acid degrading metabolism in, at least, some bacterial species (Table 3.1).

Glucose and lactate (the second most preferred energy source) along with their oxidative products (e.g. gluconate, gluconate-6-P) have been proposed to serve as spoilage indicators. This is particularly evident in the case of meat stored under aerobic conditions where pseudomonads are the major spoilage micro-organisms. Pseudomonads catabolize sequentially D-glucose and L- and D-lactic acid, with the oxidation of glucose and glucose-6-P via the extracellular pathway leading to a transient accumulation of D-gluconate and an increase in gluconate-6-P concentration. Furthermore, it has been shown that the sum of free amino acids along with the water-soluble protein content increased during storage and this corresponded well with colony counts, particularly in meat samples with relatively high glucose concentration (Nychas & Arkoudelos, 1990; Nychas & Tassou, 1997). In addition, the rate of free amino acid increase under aerobic conditions was higher than under modified atmosphere storage. These observations could have a commercial importance, since spoilage is usually associated only with post-glucose utilization of amino acids by pseudomonads (Gill, 1986).

Table 3.1 Order of substrate utilization during growth of major muscle spoilage bacteria under aerobic condition (based on Nychas, Skandamis, Tassou, & Koutsoumanis, 2008; Nychas, unpublished data)

Substrate	A	B	C	D	E	H
Glucose/glucose-6-P	1	1	1	1	1	1
Lactate	2	2		2	2	
Pyruvate	3	3				
Gluconate/gluconate-6-P	4	4				
Propionate		5				
Formate						
Ethanol		6				
Acetate		7				
Amino acids	5	8	2	3		
Ribose			3			
Glycerol			4			

A: *Pseudomonas* spp.; B: *S. putrefaciens*; C: *B. thermosphacta*; D: *Enterobacter* spp. E: *Hafnia alvei*; H: lactic acid bacteria.
Based on Gill (1986), Nychas et al. (1998), Ellis and Goodacre (2001), Nychas (unpublished)

The key chemical changes associated with the metabolic activities of pseudomonads have been the subject of intensive study. The effect of pseudomonads' growth on various substances in sterile meat block, meat juice and gel cassette system during storage at 0, 4–5, 10 and 25°C is shown in Table 3.2 (Drosinos & Board, 1994, 1995; Tsigarida & Nychas, 2001; Tsigarida et al., 2003; Roca & Olsson, 2001). The identification of the molecules acting as precursors for the production of specific catabolic products during growth of Gram-negative bacteria in broth, model system (gel cassette or sterile meat) and in naturally spoiled meat has also been thoroughly investigated and the results

Table 3.2 Metabolic activity of pseudomonads in sterile meat block, meat juice and gel cassette system at 0, 4–5, 10 and 25°C (Drosinos, 1994; Nychas et al., 1998; Tsigarida et al., 2003)

Substrate	*P. fragi*	*P. fluorescens*	*Pseudomonas* spp.
D-Glucose	+	+	+
Glucose-6-P	+	–	+
D-Gluconate	+	+	+
Gluconate-6-P	+	–	+
L-Lactic acid	+	+	+
D-Lactic acid	+	+	nd
Pyruvate	+	+	nd
Acetic acid	+	+	+
Formic acid	nd	nd	+
1-Propanol	nd	nd	+
Amino acids	+	+	nd
Creatine	+	–	nd
Creatinine	+	–	nd
Ammonia	+	+	+

are summarized in Table 3.3 (McMeekin, 1982; Dainty, Edwards, & Hibbard, 1985; Dainty, Edwards, Hibbard, & Marnewick, 1989; Edwards & Dainty, 1987; Edwards, Dainty, Hibbard, & Ramantanis, 1987; Stutz, Silverman, Angelini, & Levin, 1991; Schmitt & Schmidt-Lorenz, 1992b; Jackson, Acuff,

Table 3.3 Factors affecting the production of end products of Gram-negative bacteria (e.g. *Pseudomonas* spp., *Shewanella putrefaciens*, Moraxella) when inoculated in broth, model system (gel cassette or sterile meat) and in naturally spoiled meat in comparison with sterile meat (Tsigarida et al., 2003; Nychas et al., 1998; Nychas, unpublished)

	Conditions studied				
End product	Broth	Model food	Meat	Sterile meat	Factors
Gluconate	+	+	+	ND	Glucose and oxygen (limitation)
Gluconate-6-P	+	+	+	+	
Lactic acid	+	+	+	+	
Acetic acid	+	+	+	ND	
Formic acid	+	+	+	NAD	
Sulphides	NAD	+	+	+	Temperature and substrate (glucose) limitation
Dimethylsulphide	NAD	+	+		
Dimethyldisulphite	NAD	+	+		
Methyl mercaptan	NAD	+	+		
Methanethiol	NAD	+	+		High pH
Hydrogen sulphide	NAD	–/+	+		ND
Dimethyltrisulphide	NAD	+	+		
Methyl esters (acetate)	NAD	+	+		Glucose
Ethyl esters (acetate)	NAD	+	+		Glucose
Acetone	NAD	+	+		ND
2-Butanone	NAD	+	+		ND
2-[2-butoxyethoxy]-ethanol, acetate	NAD			+	ND
Acetoin/diacetyl	NAD	+/–	+		ND
Diethyl benzene	NAD	+	+		ND
1-Butanol	NAD	ND		+	ND
Trimethylbenzene	NAD	+	+		ND
Toluene	NAD	+	+	+	ND
Butanal	NAD	NAD	+	+	ND
Hexane	NAD	+	+		ND
2,4-Dimethylhexane	NAD	+	+		ND
Methyl heptone	NAD	+	+		ND
2-Methylbutanal	NAD	+	+		ND
Methanol	NAD	+	+		ND
Ethanol	NAD	+	+		ND
2-Methylpropanol	NAD	+	+		ND
2-Methylbutanol	NAD	+	+		ND
3-Methylbutanol	NAD	ND	+		ND
Propanol-1	NAD	+	+	+	ND
Ammonia	NAD	+	+		Glucose

NAD: not available data; ND: not determined

Vanderzant, Sharp, & Savell, 1992; Lasta, Pensel, Masana, Rodriguez, & Garcia, 1995; Roca & Olsson, 2001; Tsigarida et al., 2003; Tsigarida & Nychas, 2001). Moreover, a wide range of volatile compounds are produced during growth of spoilage microbiota in naturally contaminated samples of meat stored chilled in air (McMeekin, 1977; Dainty et al., 1985, 1989; Molin & Tenstrom, 1986; Edwards & Dainty, 1987; Stutz et al., 1991; Jackson et al., 1992; Lasta et al., 1995; Tsigarida & Nychas, 2001; Vainonpaa et al., 2004) and are presented in Table 3.4.

The increase in D-gluconate concentration inevitably led to the proposition of a new 'hurdle' regarding the extension of meat shelf life. This new hurdle was the addition of glucose in meat and its concomitant transformation to gluconate (Gill, 1986; Lambropoulou et al., 1996) with a simultaneous decrease of the pH value due to the accumulation of oxidative products. A selective determinant on meat ecosystem may be offered by this transient pool of gluconate and the inability of the taxa participating in the microbial association to utilize this additional energy source (Nychas et al., 1998). Indeed, the addition of carbohydrates, and especially glucose, has already been suggested as a factor able to delay spoilage particularly in dark, firm, dry (DFD) meat (pH > 6.0), primarily due to the fact that the glucose content affects not only the cell density attained at the onset of spoilage (Gill, 1986) but also the metabolic products produced by the microbiota (Nychas & Arkoudelos, 1990). Meat with DFD characteristics spoils more rapidly than meat of normal pH (pH 5.5–5.8).

Pseudomonas fragi was found to catabolize creatine and creatinine under aerobic conditions and release ammonia in the growth medium, resulting in pH increase. Ammonia can also be produced by many micro-organisms,

Table 3.4 Major volatile microbial metabolites detected in naturally contaminated samples of meat stored chilled in air

1-Undecene	Benzaldehyde	*Iso*-pentyl formate
1,4-Heptadiene	Butane	Methanethiol
1,4-Undecadiene	Cadaverine	Methanol
2-Butanone	Crotonate	Methyl ethyl ketone
2-Methyl butanol	Diacetyl	Methyl mercaptan
3-Methyl butanal	Diaminopropane	Methylthioacetate
3-Methyl butanol	Dimethylsulphide	*n*-Heptanoate
3-Methyl-2-butenoate	Dimethyltrisulphide	*n*-Hexanoate
3-Methylbutanoate	Ethanol	*n*-Hexanoate
4-Methyl-benzaldehyde	Ethyl acetate	*n*-Octanoate
4-Heptanol	Hexane	*n*-Propanoate
Acetaldehyde	Hydrogen sulphide	*n*-Propanoate
Acetoin	*Iso*-butanoate	
Acetone	*Iso*-butyl acetate	
Agmartine	*Iso*-pentyl acetate	
Ammonia	*Iso*-propyl acetate	

Based on Nychas et al. (1998); Nychas et al. (2007); Nychas (unpublished)

including pseudomonads during their amino acid metabolism. Ethanol, acetone, propan-2-ol, dimethylsulphide, propan-1-ol, ethyl acetate, 2,3-butandione, acetic acid, diacetyl, hexane, heptane, pentanol, heptadiene, acetoin, octane and 2,3-butandiol are other volatile compounds that have also been detected in spoiled meat (Nychas et al., 1998).

Enterobacteriaceae family can also play a role in spoilage, provided the meat ecosystem favours its growth. They preferentially utilize glucose and glucose-6-P as carbon sources and degradation of amino acids occurs only after their depletion (Gill, 1986). Moreover, some members of this family produce ammonia, volatile sulphides, including H_2S, and malodorous amines from amino acid metabolism.

Gram-positive bacteria associated with meat storage ecosystems, apart from *B. thermosphacta*, include various *Lactobacillus*, *Carnobacterium*, *Leuconostoc*, *Lactococcus* and *Weissella* species. It has been reported that oxygen tension, glucose concentration and initial pH have a major influence on the physiology of these micro-organisms, and hence on end-product formation (Nychas et al., 1998). *B. thermosphacta* has a much greater spoilage potential than lactobacilli in both aerobic and anaerobic spoilage of meat. During aerobic growth, it utilizes glucose and glutamate but no other amino acid (Gill & Newton, 1977). Additionally, during its aerobic metabolism in media containing glucose, ribose or glycerol as the main carbon and energy source, a mixture of end products including acetoin, acetic, *iso*-butyric and *iso*-valeric acids, 2,3-butanediol, diacetyl, 3-methylbutanal, 2-methylpropanol and 3-methylbutanol, is produced (Dainty & Hibbard, 1980). The precise proportion of these end products is affected by the glucose concentration, pH and temperature (Nychas et al., 1998).

Spoilage Evaluation and Prediction

Spoilage still remains a subjective assessment, at least as far as its early signs are concerned. Although a wide range of bibliographical data are currently available concerning growth and development of various micro-organisms causing spoilage, both food scientists and food industry are in need of new techniques that will provide with rapid and reliable results and diminish the drawbacks of the traditional microbiological methods.

Aerobic standard plate count has been considered as the 'gold standard' despite the fact that the results obtained underestimate the microbial load, since a microbial fraction referred to as viable but non-culturable micro-organisms (VBNC) is simply not incorporated in the result. Moreover, this technique is laborious and time consuming and thus unsuitable for products with a short shelf life. Alternative approaches have been provided with the application of direct epifluorescent filtration technique (DEFT) and ATP bioluminescence methods. In the first case, micro-organisms are extracted from the food matrix,

concentrated onto a membrane surface, stained and simply detected and enumerated using epifluorescent microscopy, whereas in the latter the amount of ATP present is measured through the amount of light emitted when luciferin and luciferase are added. Both techniques have been successfully applied for a variety of foods (Pettipher & Rodrigues, 1982; Liberski, 1990; Qvist & Jakobsen, 1985; Walls, Sheridan, Welch, & Mcdowell, 1990; Stannard & Wood, 1983; Siragusa, Dorsa, Cutter, Perino, & Koohmaraie, 1996), but improvements are necessary in order to apply to muscle foods, as in the former case the food debris interfere and in the latter the method also determines ATP originating from non–bacterial cells. Lately, flow cytometry has been proved itself as a promising method with great potential (Holm, Mathiasen, & Jespersen, 2004; Flint, Walker, Waters, & Crawford, 2007). Possible problems will most probably arise by the food debris, as the micro-organisms will have to be successfully extracted onto a filter that then have to be scanned by a laser beam.

Recent advances in biotechnology allowed the use of genetic tools for microbial detection. PCR-based techniques, such as PCR coupled with an ELISA assay as well as real-time PCR either coupled or not with reverse transcription, have been successfully applied for the detection and estimation of the total microbial load or specific pathogen population in meat samples (Gutierrez et al., 1998; Josefsen, Krause, Hansen, & Hoorfar, 2007; Navas et al., 2006; Perelle, Dilasser, Grout, & Fach, 2007; Holicka, Guy, Kapoor, Shepherd, & Horgen, 2006). Despite the fact that they are accurate and rapid methods, their complexity and the need of highly trained personnel make them unsuitable for routine analysis.

Microbial growth and development has been rightfully correlated with the chemical changes that occur during spoilage. In that manner, changes in the concentration of substrates or determination of specific metabolic products could be used as spoilage indicators, towards the assessment of meat quality. Ideally, a substance can serve as a spoilage indicator when, among others (Jay, 1986), it

(i) is absent or at least at low levels in meat,
(ii) increases with storage,
(iii) is produced by the dominant microbiota and
(iv) shows a good correlation with scores of organoleptic tests.

A serious amount of data currently exists, concerning the potential use of certain metabolites as spoilage indicator (Table 3.5). Nonetheless, due to the lack of general agreement on the early quality changes, none of these metabolites can be used for that purpose. Furthermore, most of the proposed methods are inadequate to rapidly provide with results, whereas advances in preservation technology seem to have a negative impact on these methodologies.

Identification of an ideal metabolite that could be used for spoilage assessment relies on the fact that growth of a micro-organism will cause an ongoing decrease in the metabolizable substrates and a respective increase in the amount

Table 3.5 Compounds which may be important for the determination/prediction or assessment of the remaining shelf life of raw meat under different conditions

Compound	Method	Storage conditions	Literature
Low molecular compounds, e.g. glucose, acetate, gluconate, lactate	Enzymatic kit, HPLC	Air, vp-map	Nychas, Dillon, and Board (1988) Nychas and Arkoudelos (1991) Borch and Agerhem (1992) Kakouri and Nychas (1994) Nassos, King, and Stafford (1983, 1985, 1988)
Ethanol	Enzymatic kit, GLC	vp-map	Dainty (1996)
Ammonia	Enzymatic, colorimetric	Air	Nychas and Arkoudelos (1991) Nychas (1984)
Methyl acetone	GLC	vp-map	Stutz et al. (1991)
Ethyl ketone	GC/MS		Vainonpaa et al. (2004)
Dimethyl sulphide	Sulphur selective detector		
Dimethyldisulphide			
Hydrogen sulphide			
diacetyl, acetoin	Colorimetric	vp-map	Ordonez, de Pablo, Perez de Castro, Asensio, and Sanz (1991) Nychas and Arkoudelos (1991)
Biogenic amines	HPLC, sensors, enzymic test, GLC Enzyme electrodes, test strips	Air, vp, map	Schmitt and Schmidt-Lorenz (1992a) Yano, Kataho, Wataanabe, and Nakamura (1995) Rokka et al. (2004)
Diamines	Amperometric electrodes (enzymatic systems)	Air	Ellis and Goodacre (2001)
Microbial activity	Enzymic	Air	Seymour, Cole, and Coote (1994) Alvarado, Rodriguez-Yunta, Hoz, Garcia de Fernando, and Ordonez (1992)

Table 3.5 (continued)

Compound	Method	Storage conditions	Literature
Volatiles (odours)	Electronic noses (chemical sensors)	Air-vp-map	Ellis and Goodacre (2001); Meyer, Larsen, and Revsbech (2002)
Proteolysis (Amides, amines, etc.)	Fourier transform infrared spectroscopy (FT-IR) Electrophoresis HPLC	Air-vp-map—active packaging	Ellis, Broadhurst, Kell, Rowland, and Goodacre (2002) Goodacre, Vaidynathan, Dunn, Harrigan, and Kell (2004) Ammor, Argyri, and Nychas (2008) Schmitt and Schmidt-Lorenz (1992b) Nychas and Tassou (1997)

of the metabolic end products. This has proved to be a difficult task due to the following reasons:

(i) most metabolites are specific to certain organisms (e.g. gluconate to pseudomonads). Thus, such an indicator would provide with inaccurate spoilage information when the respective micro-organisms are not present or inhibited by the natural, or imposed food microbiota,
(ii) absence of a given substrate able to support microbial growth, or even presence in low quantities does not necessarily preclude spoilage,
(iii) natural or imposed, intrinsic or environmental conditions affect the rate of metabolite production as well as the metabolic pathways of micro-organisms,
(iv) accurate detection and measurement of metabolites are usually laborious and time-consuming, and
(v) the retrospective information provided by many of the metabolites is simply not satisfactory.

Recently, a variety of techniques aiming at the, more or less, indirect estimation of spoilage have emerged, among them, electrical methods, i.e. methods detecting the changes in the impedance and conductance of the growth medium caused by the utilization of metabolizable molecules during microbial growth. With the application of such methods, microbial growth can be detected as changes in the flow of current. Moreover, spectroscopic methods such as Fourier transform infrared spectroscopy (FT-IR) as well as short wavelength near-infrared diffuse reflectance spectroscopy (SW-NIR) have proven their capacity as spoilage detection methods (Ellis, Broadhurst, & Goodacre, 2004; Lin et al., 2004; Lin, Mousavi, Al-Holy, Cavinato, & Rasco, 2006). With the application of both techniques, a spectrum is received, depicting, more or less, the chemical composition of the sample. Spoilage can be detected via the comparison with control samples using advanced statistical methods (discriminant function analysis, clustering algorithms, chemometrics) and intelligent methodologies (neural networks, fuzzy logic, evolutionary algorithms and genetic programming). Electric nose has also been used to assess and predict spoilage in a, so far, limited range of foodstuffs (Blixt & Borch, 1999; Du et al., 2001) through the detection of volatile compounds produced during spoilage and can also be used to identify particular species of micro-organisms with a unique volatile fingerprint.

When quantitative evaluation of spoilage and safety is under study, there is a variety of factors that should be taken into consideration, among them, food structure (i.e. matrix effects) and physicochemical parameters (e.g. type, concentration and availability of nutrients), interactions among the microbiota that form the respective microenvironment, aspects concerning the microbial physiology as well as effects of dynamic storage conditions (fluctuation of temperature, packaging in vp/map, film permeability, etc.). Thus, by understanding the mechanisms involved in the development of the particular micro-ecosystem and the effect on the expression of certain genotypes, we will be able

to know when and how to exploit not only the produced catabolites but also the respective substrates as well for the benefit of the industry, regulatory authorities and consumers. Meat industries as well as inspection authorities need rapid analytical methods and tools, the former for quantification of these indicators in order to determine what kind of processing is suitable for their raw materials and to predict the remaining shelf life of their products and the latter for control purposes. Retail and wholesale also need these valid methods to ensure the freshness and safety of their products to resolve disputes between buyers and sellers. Reliable indication of the safety and quality status of meat at retail and until consumed is desirable. It is therefore crucial for the development of valid methods to monitor freshness and safety and overall quality, regardless of the perspective (i.e. that of the consumer, the industry, the inspection authority or the scientist).

Quantitative estimation of growth of ESOs led to the development of mathematical models able to describe spoilage and on the other hand predict the shelf life of meat. Such models describing growth of *B. thermosphacta*, *Pseudomonas* spp. and *S. putrefaciens* in aerobically stored and CO_2-packed raw meat and fish have been successfully validated for shelf life prediction. Moreover, stochastic models that take into account the distribution of spoilage bacteria on products and the storage temperature have been developed for shelf life prediction of fresh aerobically stored fish (Koutsoumanis, 2001; Giannakourou, Koutsoumanis, Nychas, & Taoukis, 2001; Koutsoumanis et al., 2002; Rasmussen, Ross, & McMeekin, 2002). However, there is a limited number of such models that have been successfully validated for the growth of ESOs and have been included in application software facilitating prediction of food shelf life under constant and dynamic temperature storage conditions (Combase; www.combase.cc; Koutsoumanis and Nychas Spoilage Predictor, submitted patent). Apart from models for ESOs, progress on predicting modelling under dynamically changing temperatures has also been made for pathogens, e.g. *Salmonella*, *Listeria Monocytogenes* (Bovill, Bew, &Baranyi, 2001). The construction of models to predict the development of microbial spoilage associations in new formulations of lightly preserved seafood remains an important challenge in the field of meat microbiology. It has been shown that this approach can be used to predict, with confidence, the effects of environmental variables, such as temperature, water activity and pH on the growth and survival of bacteria in foods (McClure, 1994). The effect of carbon dioxide has been modelled primarily in liquid media (Farber, Cai, & Ross, 1996), whereas, regarding meat, two models have been published for the growth of *L. monocytogenes* in cooked meat products (Duffy, Vanderlinde, & Grau, 1994; Devlieghere et al., 2001), with one including carbon dioxide as controlling factor (Devlieghere et al., 2001). Farber et al. (1996) modelled the effect of carbon dioxide (10–90%), pH (5.5–6.5) and temperature (4–10°C) on the growth of *L. monocytogenes* in brain heart infusion broth. A good agreement has also been obtained between predictions and observed growth of *L. monocytogenes* in modified atmosphere packed foods, including many meat products (Duffy et al., 1994; Devlieghere et al., 2001). In the latter

products, effects of additional factors, such as sodium lactate, nitrites and ascorbate, have also been included.

Finally, recent attempts have been made to evaluate the risk of consumption of meat and meat products contaminated with pathogens, especially *Escherichia coli* O157:H7 and *L. monocytogenes* in order to facilitate the implementation of Hazard Analysis Critical Control Points (HACCP) systems in meat industry (FDA/CFSAN, 2003). According to the available literature data on meat (specifically ground beef), the risk assessments that have been conducted for *E. coli* O157:H7 on hamburgers aimed either to identify data gaps in evaluating the risk of illness by consumption of contaminated and improperly cooked hamburgers (Marks, Coleman, Lin, & Roberts, 1998) or to model the exposure of consumers to this pathogen from farm to fork (Cassin, Lammerding, Todd, Ross, & McColl, 1998).

Strategies for Spoilage Control

Expansion of muscle food shelf life can be achieved using a variety of approaches and strategies. Storage at chill temperatures ($<5^{\circ}$C) can expand the shelf life for days. Modified atmosphere or vacuum packaging storage can achieve it for weeks or months. Further expansion requires the use of more drastic measures such as canning, freezing, use of chemical or biological preservatives. Given that the move towards minimally preserved foods is increasing, the most attractive and promising methodology is modified atmosphere or vacuum packaging or even active and intelligent packaging.

Meat packaging technologies aim at multiple targets, with the most important being shelf life expansion, improvement of product appearance and presentation, reduction of other preservatives, minimization of meat waste and accession of new markets.

A modified atmosphere can be defined as one that is created by altering the natural composition of air (78% nitrogen, 21% oxygen, 0.03% carbon dioxide and traces of noble gases) to provide an alternative atmosphere for increasing storage time and quality of food/produce (Phillips, 1996). Modified atmosphere packaging (MAP) can be distinguished into two types: active and passive atmosphere modification. The former is achieved with the displacement of gases in the package, and their replacement by a desired mixture of gases, while regarding the latter, the product is packaged using a selected film type, and a desired atmosphere develops naturally as a consequence of either the products' respiration or the diffusion of gases through the film (Moleyar & Narasimham, 1994; Zagory, 1999; Lee, Sebranek, & Parrish, 1996). Carbon dioxide, oxygen and nitrogen are mainly used in MAP preservation of meat. Carbon dioxide is used as an inhibitor of most bacteria and mould growth, oxygen as an inhibitor of anaerobic micro-organisms, mainly in some types of fish and vegetables, while maintaining fresh and natural colour in red meats and respiration in fruits and

vegetables. Finally, nitrogen is used to exclude air and to prevent the collapse of packs for high-moisture and fat-containing foods. These gases are usually combined in three ways: inert blanketing using N_2, semi-reactive blanketing using CO_2/N_2 or $O_2/CO_2/N_2$ or fully reactive blanketing using CO_2 or CO_2/O_2 (Moleyar & Narasimham, 1994). Other gases, such as carbon monoxide, nitrous and nitric oxides, sulphur dioxide, chlorine and ozone (Phillips, 1996), have also been used, but mostly experimentally due to safety and regulatory reasons along with cost restrictions and considerations.

Meat colour is an important aspect and the principal quality characteristic affecting a consumer's decision. To avoid red meat discolouration in MAP, a high pO_2 is included so that the oxygenated bright red colour (i.e. oxymyoglobin) is retained; a method developed to achieve this objective is called 'high oxygen modified atmosphere' (Gill & Molin, 1991). There is, however, a relationship between oxygen and carbon dioxide in a meat system, meat quality and shelf life, in general. Both gases play an important role in selecting different microbial associations from the initial contaminants (oxygen an aerobic and carbon dioxide a facultatively anaerobic flora) and both influence the meat colour in different ways. A high pO_2 retains an acceptable colour but causes oxidation of fat and, thus, product rancidity is enhanced. On the other hand, carbon dioxide is deleterious to colour due to protein denaturation and surface bleaching. The effect of $CO–CO_2–N_2$ (0.4% CO/60% CO_2/40% N_2), $O_2–CO_2$ (70% O_2/30% CO_2) and $CO_2–N_2$ (60% CO_2/40% N_2) on the shelf life and colour of ground beef, beef loin steaks and pork chops stored at 4 or 8°C has been studied by Sorheim, Nissen, and Nesbakken (1999), concluding that CO mixtures resulted in the highest shelf life, while they maintained longer the bright red colour of meat compared to high O_2 mixtures.

The successful application of modified atmosphere packaging requires special attention on the following issues:

(i) The extent of the initial contamination. Considerable initial population imperil the successful application of new packaging technologies.
(ii) The time of application. The earlier the selection of an association by extrinsic factors begins, the better the results that can be anticipated.
(iii) Temperature control is of vital importance due to its selective action on the ecosystem.
(iv) The gas mixture selected should be chosen according to the particular ecosystem and the effect on the colour of the meat.
(v) The permeability of the different packaging materials to the gases used should be selected critically so that the added gases or indeed those produced de novo in an ecosystem are retained.
(vi) Combination processes (e.g. MAP-irradiation) or the packaging technologies alone change the spoilage pattern of the ecosystem.

Red meats and poultry come from warm-blooded animals and, as such, their microbial flora is heterogeneous, consisting of mesophilic and psychrotrophic bacteria, including pathogenic species originating either from the animal itself

or from the environment, whereas several bacterial species can be introduced during slaughter and processing of raw products.

The principal pathogens of concern are *Aeromonas hydrophila, L. monocytogenes, Yersinia enterocolitica*, *Salmonella* spp., enterohaemorrhagic *E. coli*, *Campylobacter jejuni/coli, Staphylococcus aureus, Clostridium perfringens* and *Clostridium botulinum* (mainly processed products). Research has mainly focused on *A. hydrophila*, *Y. enterocolitica, Salmonella* spp., *E. coli* and *L. monocytogenes* and the main conclusions are discussed below.

All data available concur that *A. hydrophila* is inhibited in MAP. Thus, this micro-organism should not be considered as an added hazard in meat products packaged in modified atmospheres especially when temperatures are kept low.

L. monocytogenes is ubiquitously found in environments such as the gastrointestinal tract of both healthy humans or animals, household environments (refrigerators and cleaning cloths), food products, soil, water and silage or other decaying vegetation. Meat and fish products can be considered as hazardous because this bacterium is often present in these foods and has the potential to survive and multiply under cold or abusive storage conditions (Buchanan, Stahl, & Whiting, 1989). The effect of MAP in growth of this micro-organism is rather unclear. Experiments performed by Wimpfhimer, Altman, and Hotchkiss (1990) exhibited that its growth was induced when oxygen was added to atmospheres in which it was previously suppressed, whereas no difference was observed in its growth in pork at 4°C packaged in MAP in the absence or in the presence of reduced amount of oxygen. Furthermore, no growth was observed in either unpackaged or packaged in modified atmospheres minced meat at 4°C (Johnson, Doyle, & Cassens, 1988; Shelef, 1989) or 25°C (Shelef, 1989). The generation time (g) values of pork and turkey native microbiota were measured when they were packaged in modified atmospheres (Mano et al., 1995). It has been concluded that the generation time values were greater for *Listeria* than for the other members of the microbiota, and these values increased as the CO_2 concentration increased indicating therefore the inhibitory effect of CO_2. It is generally accepted that the atmospheres in which *L. monocytogenes* multiplication is inhibited are not bactericidal and the cases in which a decrease in *Listeria* counts was observed during storage were only of little relevance (Mano et al., 1995). Thus, control of *Listeria* spp. population cannot be achieved by mere modification of the atmosphere, and additional factors such as temperature, pH and even possibly competition with other micro-organisms should be accordingly addressed. Therefore, packaging in modified atmospheres does not necessarily signify additional hazards of *L. monocytogenes* growth in comparison to conventional packaging in aerobic conditions.

Y. enterocolitica and related species are present in all terrestrial and fresh water ecosystems. Pork products are possibly the most important foodstuff with the potential of transmitting *Yersinia* spp. to humans. Parameters such as product pH, storage temperature and other environmental factors including MAP greatly affect its growth and survival (Nychas, 1994). Although the generally accepted minimum growth temperature is 4°C (Varnam & Evans,

1991), temperatures as low as 1, –1.5 and even –2°C have also been found to support its growth (Hanna, Stewart, Zinc, Carpenter, & Vanderzant, 1977; Hudson, Mott, & Penney, 1994; Gill & Reichel, 1989). Taking under consideration all the experiments to date on this organism, definite conclusions cannot yet be drawn and thus more research is recommended, especially as far as the effect of pH, temperature and packaging in modified atmospheres on growth of this bacterium is concerned.

Salmonella spp. is not considered a psychrotrophic pathogen and thus only a limited number of studies currently exist regarding its growth under different packaging methods. *Salmonella* spp. survival and growth are well known to depend, besides temperature, on numerous factors such as pH (acid-tolerant bacterium), atmosphere and competitive flora. It has been exhibited that *S*. Enteritidis is able to survive during storage at 3°C under several atmospheres of inoculated poultry having different pH values (breast—low pH and thigh—high pH). On the contrary, storage at 10°C resulted in a rapid increase of the numbers of *S*. Enteritidis regardless of the composition of flushed air with the = exception of those samples that were stored under 100% CO_2, in which the numbers of *S*. Enteritidis decreased (about 1 log unit) after 12 days storage in breast, while in thigh (lower leg) meat they remained at the initial level. Similar results were obtained by Gray, Elliot, and Tomlins (1984). No doubt this can be attributed to the higher pH of thigh meat, which could be considered as a type of DFD muscle. This study demonstrates that temperature abuse occurring in the commercial chain of food handling may constitute a risk, as far as growth of *S*. Enteritidis is concerned. This situation may as well occur with other non-psychrotrophic organisms.

A gap in the literature is also evident as far as *E. coli* O157:H7 survival under MAP, or vacuum, is concerned (Duffy, Garvey, & McDowell, 2001). On the other hand, numerous studies have investigated the survival and acid tolerance response of *E. coli* O157:H7 during manufacturing and storage of processed meat products under vacuum, such as pepperoni (Glass, Loeffelholz, Ford, & Doyle, 1992; Riordan et al., 2000), indicating the increased resistance of this pathogen to acidic ecosystems related to meat processing. It has been reported (Van Netten, Valentijn, Mossel, & Huis in't Veld, 1998) that lactic acid (up to 5%) decontamination of pork belly cuts resulted in a delay of *E. coli* O157:H7 growth at 12.5°C aerobically, until the pH of meat surface was raised above 4.8, regardless of previous adaptation to acid. It is generally suggested that the behaviour kinetics of *E. coli* O157:H7 under MAP or vacuum are similar to those under aerobic conditions (Hao & Brackett, 1993). Moreover, storage temperature has been concluded to be a more crucial factor than packaging atmosphere, in terms of *E. coli* O157:H7 survival on fresh produce (Hao & Brackett, 1993).

The contradictory bibliographical information concerning the ability of vacuum or MAP to control pathogens may be due to the fact that in such systems emphasis has been given to the concentration of CO_2 rather than to the ability of the packaging film to maintain the flushed gaseous composition

(Stanbridge & Davies, 1998). Therefore, a few additional significant issues should be addressed. In general, such studies take into consideration variables, either alone or in combination (e.g. *Pseudomonas* and *Listeria* or *Pseudomonas* and film permeability), but seem to underestimate or neglect the interactions that give rise to new variables that concomitantly emerge (Tsigarida, Skandamis, & Nychas, 2000; Skandamis, Tsigarida, & Nychas, 2002). For example, the composition of the gaseous atmosphere within VP/MAP dramatically changes when O_2-permeable packs are used (McMullen & Stiles, 1991) allowing thus growth of pseudomonads (Newton & Rigg, 1979; Tsigarida & Nychas, 2001), which in turn seem to enhance the growth of *L. monocytogenes* (Tsigarida et al., 2000). Similar observations have been reported for beef, milk and re-cooked chicken nuggets (Marshall & Schmidt, 1988; Farrag & Marth, 1989; Marshall, Andrews, Wells, & Farr, 1992). Growth of *L. monocytogenes* in milk has been attributed to protein hydrolysis by pseudomonads that provide with free amino acids (Marshall & Schmidt, 1991). Proteolysis caused by the microbial association and *P. fragi* was also evident in chicken breast stored under aerobic, VP and MAP conditions (Nychas & Tassou, 1997), whereas the fact that pseudomonads did not release such nutrients on endive leaves could be the reason for the lack of stimulation of *L. monocytogenes* growth (Carlin, Nguyen, & Morris, 1996). On the other hand, pseudomonads may inhibit the growth of *L. monocytogenes*. This was the case of sterile minced beef medium and has been assigned to competition for nutrients (Mattila-Sandholm & Skytta, 1991). In the case of meat stored under VP and MAP in low-permeability film, *B. thermosphacta* constituted the major proportion of the total microbiota and no growth of *L. monocytogenes* was detected on either naturally contaminated or sterile meat fillets (Tsigarida et al., 2000).

The ability of the micro-organisms to adapt to an environment is found to be of fundamental importance for their survival and growth. Indeed, growth of a bacterium under an imposed stress (e.g. low pH, temperature, preservative, nutritional or oxygen limitation) before contaminating some food ecosystem exhibited that multiplication of the micro-organism was evident, and furthermore such stressful conditions could initiate growth in a refrigerated food more rapidly than in optimal environments. This was the case of *L. monocytogenes* (Buchanan & Klawitter, 1991; Gay, Cerf, & Davey, 1996) and *E. coli* (Van Netten et al., 1998) as the inhibitory effects in raw-ground beef were absent in cooked beef. Grau and Vanderlinde (1992) found a 53% incidence of listeriae in a survey of vacuum-packaged processed meats. Growth of *L. monocytogenes* in the presence of the native microbiota (lactic acid bacteria and *B. thermosphacta*) on vacuum-packaged corned beef and ham contaminated naturally or deliberately with the pathogen was also investigated. The combined effect of temperature, pH, salt, a_w and residual nitrite on the growth of *L. monocytogenes* was noted and it was concluded that these ecological determinants influenced markedly the growth pattern of the pathogen on chilled meats. Gas-modified atmosphere packaging of fresh pork chops has been reported to hinder the growth of *L. monocytogenes* as well as that of the autochthonous microbiota

(Manu-Tawiah, Myers, Olson, & Molins, 1993). Moreover, the organoleptic changes occurred may not alert a consumer of the hygienic status of the product under the imposed conditions. On the contrary, no inhibition of *L. monocytogenes* and on cooked poultry stored under MAP in the presence of the native microbiota was observed (Barakat & Harris, 1999), while the latter seemed to inhibit growth of *Y. enterocolitica* on minced beef (Kleinlein & Untermann, 1990).

The combined effect of microbial interaction with abiotic factors (e.g. modified atmosphere), has been thoroughly investigated by Hintlian and Hotchkiss (1987); cooked beef was inoculated with (i) *P. fragi* and *C. perfringens*, (ii) *P. fragi*, *Salmonella* Typhimurium and *S. aureus* or (iii) *P. fragi, C. perfringens, S.* Typhimurium and *S. aureus* and stored the samples at different temperatures. The conclusion drawn was that an atmosphere containing 75% CO_2, 15% N_2 and 10% O_2 was the most effective for growth control of both the pseudomonads and the pathogens. Interestingly, extensive growth of *C. perfringens* (situation (iii) above) at 12.8°C was observed most probably due to the consumption of oxygen by pseudomonads.

Over the last few years, the need for the development of novel food packaging concepts that can either play an active role in product preservation or even assist towards product improvement has been recognized. A variety of different approaches have been applied and despite occasional inconveniences, many interesting packaging technologies have been created. Among them oxygen scavengers, carbon dioxide scavengers and emitters, moisture controllers and antimicrobial agents have been successfully incorporated in the packaging materials.

Oxygen scavenging technologies utilize one or more of the following concepts: iron powder oxidation, ascorbic acid oxidation, photosensitive dye oxidation, enzymatic oxidation (e.g. glucose oxidase and alcohol oxidase), unsaturated fatty acids (e.g. oleic or linolenic acid), rice extract or immobilized yeast on a solid substrate (Floros et al., 1997) in order to control oxygen levels within the packaging. Such scavenging films have applications in a wide variety of food products including dried or smoked meat products and processed meats (Kerry, O'Grady, & Hogan, 2006).

Carbon dioxide is added in a packaging environment to suppress microbial growth; therefore, a carbon dioxide generating system can be viewed as a technique complimentary to oxygen scavenging (Suppakul, Miltz, Sonneveld, & Bigger, 2003). As the permeability of carbon dioxide is much higher than that of oxygen in most plastic films, the desired concentration can only be maintained when carbon dioxide is continuously produced (Ozdemir & Floros, 2004). Apart from its desirable effects on foods such as meat and poultry, in terms of surface microbial growth inhibition and consequent shelf life expansion, a carbon dioxide emitter is desirable as it prevents collapse of flexible packaging. Carbon dioxide absorbers (sachets), consisting of either calcium hydroxide and sodium hydroxide or potassium hydroxide, calcium oxide and silica gel, may be used to remove carbon dioxide during storage to prevent

bursting of the package. Possible applications include their use in packs of dehydrated poultry products and beef jerkey (Ahvenainen, 2003).

Moisture controllers consist of an absorbent polymer located between two layers of a micro-porous or non-woven polymer. Their main purpose is to lower the water activity of the product, thereby preventing surface microbial growth. Such sheets are used as drip-absorbing pads placed under whole chickens or chicken cuts.

Direct incorporation of antimicrobial agents in packaging materials is a convenient means by which antimicrobial activity can be achieved. The incorporation of chemical preservatives in the packaging material has also been the subject of intensive study. Ouattara, Simard, Piette, Begin, and Holley (2000) reported the effective inhibition of surface spoilage bacteria in processed meats following the application of antimicrobial films prepared by incorporating acetic or propionic acid into a chitosan matrix, with or without addition of lauric acid or cinnamaldehyde. Although lactic acid bacteria were unaffected by the antimicrobial films studied, growth of surface inoculated onto the meat products Enterobacteriaceae and *Serratia liquefaciens* was delayed or completely inhibited as a result of film application. On the other hand, 1.0% triclosan film did not effectively reduce spoilage bacteria and growth of *L. monocytogenes* on refrigerated vacuum-packaged chicken breasts stored at 7°C (Vermeiren, Devlieghere, & Debevere, 2002).

A new insight has been provided by the concept of intelligent packaging that is based on the development of non-invasive microbial growth sensors and indicators to monitor the spoilage level of food products. From that point of view, gas sensors, i.e. devices that respond quantitatively to the presence of gaseous analyte, can be used either as a leakage indicator or to verify the efficiency of an oxygen scavenger, a carbon dioxide scavenger/emitter or even the combination of these gases using phase fluorimetric detection (Neurater, Klimant, & Wolfbeis, 1999). Other approaches to freshness indication, which may find commercial application in intelligent meat packaging systems, are those based on the recently developed biosensor technologies, i.e. analytical devices that detect, record and transmit information pertaining to biological reactions (Yam, Takhistov, & Miltz, 2005). Despite the need for experimentation, a visual diagnostic system that incorporates antibodies in a polyethylene-based plastic packaging and is capable of detecting *Salmonella* spp., *Campylobacter* spp., *E. coli 0517* and *Listeria* spp. has been developed (Bodenhammer, 2002; Bodenhammer, Jakowski, & Davies, 2004). Package integrity has also been the target for the development of specific indicators, on the basis that package integrity is an essential requirement for the maintenance of quality and safety standards in packaging of meat products. Research has rightfully focused on the development of visual oxygen indicators in MAP foods, with the exception of the high oxygen content MA packaging of fresh meat. The outcome of this was a series of visual oxygen indicators consisting mainly of redox dyes that have been patented (Davies & Gardner, 1996; Krumhar & Karel, 1992;

Mattila-Sandholm, Ahvenainen, Hurme, & Jarvi-Kaarianen, 1995; Yoshikawa, Nawata, Goto, & Fujii, 1987).

The chemical changes during spoilage have been the target of the freshness indicators, on the basis of the reactions between indicators included within the package and microbial growth metabolites (Smolander, 2003). A number of characteristic metabolites associated with muscle food products exist upon which indicator development may be based. Changes in the concentration of organic acids such as n-butyrate, L-lactic acid, D-lactate and acetic acid, ethanol as well as carbon dioxide during storage indicate microbial growth and offer potential as indicator metabolites for a number of meat products (Shu, Hakanson, & Mattiason, 1993; Randell et al., 1995). In addition, biogenic amines such as histamine, putrescine, tyramine and cadaverine have been implicated as indicators of meat product decomposition (Kaniou, Samouris, Mouratidou, Eleftheriadou, & Zantopoulos, 2001; Okuma, Okazaki, Usami, & Horikoshi, 2000; Rokka, Eerola, Smolander, Alakomi, & Ahvenainen, 2004). Their lack of impact on sensory quality along with the toxicological concerns associated with these compounds exhibits the importance of an effective amine indicator. Such detection systems described by Miller, Wilkes, and Conte (1999) and Loughran and Diamond (2000) provide potential for commercial development. Finally, an approach based on the fact that the breakdown in the chill chain during distribution is a major factor contributing in the spoilage of fresh muscle has also been proved helpful. Time–temperature integrators are small and inexpensive devices that can be incorporated into a food package and notify of the time and temperature history of the stored product. These devices are very useful, as temperature abuse can be understood even by untrained persons, such as consumers.

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Chapter 4
Mycotoxins in Meat and Processed Meat Products

Jean-Denis Bailly and Philippe Guerre

Introduction

Mycotoxins are toxic substances elaborated by fungi. They constitute a heterogeneous group of secondary metabolites with diverse potent pharmacological and toxic effects in humans and animals. More than 300 secondary metabolites have been identified but around 30 are of real concern for human and animal health (for review, see Bennett & Klich, 2003). These molecules are produced during mould development on plants in the field or during storage period. They can be found as natural contaminants of many vegetal foods or feeds, mainly cereals, but also fruits, nuts, grains, forage as well as compound foods intended for human or animal consumption. Most important mycotoxins are produced by moulds belonging to *Aspergillus*, *Penicillium* and *Fusarium* genus (Bhatnagar, Yu, & Ehrlich, 2002; Conkova, Laciakova, Kovac, & Seidel, 2003; Pitt, 2002). These molecules are usually classified depending on the fungal species that produce them (Table 4.1).

If some toxins display an important acute toxicity (after unique exposure to one high dose), chronic effects (observed after repeated exposure to weak doses) are probably more important in humans. Mycotoxins are suspected to be responsible for several pathological syndromes in human: ochratoxin A and Balkan endemic nephropathy, oesophageal cancer and fumonisin B1, etc. Mycotoxin exposure of human consumers is usually directly linked with alimentary habits.

Mycotoxin toxicity is variable (Table 4.2). Some are hepatotoxic (aflatoxins), immunotoxic (trichothecenes, fumonisins), others have an estrogenic potential (zearalenone), etc. (Bennett & Klich, 2003). Certain mycotoxins are considered as carcinogenic or suspected to have carcinogenic properties (IARC, 1993).

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Table 4.1 Mycotoxins and producing fungal species associated with human and animal nutrition

Mycotoxins	Main producing fungal species
Aflatoxins B1, B2, G1, G2	*Aspergillus flavus, A. parasiticus, A. nomius*
Ochratoxin A	*Penicillium verrucosum, Aspergillus ochraceus, Aspergillus carbonarius*
Fumonisins B1, B2, B3	*Fusarium verticillioides, F. proliferatum*
Trichothecenes	*Fusarium graminearum, F. culmorum, F. sporotrichioides, F. poae, F. tricinctum, F. acuminatum*
Zearalenone	*Fusarium graminearum, F. culmorum, F. crookwellense*
Patulin	*Penicillium expansum, Aspergillus clavatus, Byssochlamys nivea*
Ergot alkaloids	*Claviceps purpurea, C. paspali, C. africana*
Citrinin	*Aspergillus terreus, A. carneus, A. niveus, Penicillium verrucosum, P. citrinum, P. expansum*
Cyclopiazonic acid	*Aspergillus flavus, A. versicolor, A. tamarii, Penicillium camemberti*
Sterigmatocystin	*Aspergillus flavus, A. versicolor, A. nidulans*
Sporidesmins	*Pythomyces chartarum*
Stachybotryotoxins	*Stachybotrys chartarum, S. atra*
Endophyte toxins	*Neotyphodium coenophialum, N. nolii*
Tremorgenic toxins	*Penicillium roqueforti, P. crustosum, P. puberrelum, Aspergillus clavatus, A. fumigatus*

For human consumers, the main source of exposure to mycotoxins is cereals and cereal-based products (Leblanc, 2004; Schothorst & Van Hegmond, 2004; SCOOP Report, 2000). However, human consumers may also be exposed to these toxic compounds indirectly due to the presence of residual contamination

Table 4.2 Toxic effects of main mycotoxins; cellular and molecular mechanisms of action

Toxin	Toxic effect	Mechanism of action
Aflatoxin B1 and M1	Hepatotoxic Genotoxic Carcinogenic Immunomodulation	Bioactivation by P450 cytochromes Lipids peroxydation Formation of DAN adducts
Ochratoxin A	Nephrotoxic Genotoxic Immunomodulation	Effect on protein synthesis Inhibition of ATP production
Trichothecenes (T-2 toxin, DON, etc.)	Haematotoxicity Immunomodulation skin toxicity	Impact on protein synthesis Apoptosis of haematopoietic stem cells and on immune cells Alteration of immunoglobulin
Zearalenone	Fertility and reproduction troubles	Bioactivation by reductases Link to estrogenic receptors
Fumonisin B1	Lesion of central nervous system Haematotoxicity Genotoxicity Immunomodulation	Inhibition of ceramide synthesis Modification of sphinganine/sphingosine ratio Alteration of cell cycle

in foods prepared from animals that have been fed with contaminated feeds. Depending on the metabolic pathways involved, the residues may correspond to the native toxin or to metabolites that keep all or part of the toxic properties of the parental molecule. Therefore, the passage through an "animal filter" may represent a detoxification process or, on the contrary, lead to the appearance of more toxic compounds for the human consumer.

Therefore, animal-derived products such as meat, eggs and milk may represent a vector of mycotoxins. Moreover, the great stability of these compounds allows them to resist to classical process of cooking and/or sterilization (Park, 2002; Ryu, Jackson, & Bullerman, 2002).

The exposure of human consumers may also result in the mycotoxin synthesis during ripening of products. Indeed, several studies have shown that mould species belonging to the genus *Penicillium* and *Aspergillus* could be isolated from meat products such as ripened sausages or dry cured ham (Andersen, 1995; Leistner, 1990; Tabuc, Bailly, J.D., Bailly, S., Querin, & Guerre, 2004). This mycoflora actively participates in the acquisition or improvement of organoleptic qualities of these products. However, fungal development also raises the question of a possible mycotoxin synthesis on these substrates, leading to the contamination of final products. Usually, fungal ferments used are selected for their lack of toxigenic potential (*P. nalgiovensis* for instance). However, many studies demonstrated that fungal mycoflora of dry cured meat products is usually complex and made of many fungal species, from which several may be toxinogenic, at least in vitro. Therefore, the contamination with toxigenic strain may lead to mycotoxin synthesis and accumulation in the final product (Bailly, Tabuc, Querin, & Guerre, 2005).

At the present time, only aflatoxins and, to a lesser extent, ochratoxin A are regulated in foods from animal origin. For other toxins, the risk management is based on the control of the contamination of food from vegetal origin intended for both human and animal consumption. Regulatory values or recommendations are mainly built on available knowledge on toxicity and potential carry-over of these molecules in animal. Therefore, by limiting animal exposure through feed ingestion, one can guarantee against the presence of residues of mycotoxins in animal-derived products. However, accidental high levels of contamination may lead to a sporadic contamination of products coming from exposed animals.

The aim of this work is to present the available data on mycotoxin contamination of meat and processed meat products. Due to the important structural diversity of mycotoxins and the variations in their metabolism, it is impossible to edit general rules and each toxin and each product has to be investigated as a particular case. Therefore, presentation will be made toxin by toxin, presenting successively the origin and toxicological features of each molecule and the available data on possible contamination of meat and processed meat products, respectively.

Aflatoxins

Introduction

Aflatoxins are the most documented mycotoxins. They were discovered following a toxic accident in Turkeys fed with groundnut oilcake supplemented diet (Turkey X disease) (De Iongh, Berthuis, Vles, Barrett, & Ord, 1962; Nesbitt, O'Kelly, Sargeant, & Sheridan, 1962). The toxicity of these molecules was then studied in many animal species. Moreover, it has been demonstrated that aflatoxin B1 ingested by dairy cows was partially metabolized into a molecule called "milk aflatoxin 1" (AFM1) (Allcroft & Carnaghan, 1963). These studies demonstrated that mycotoxins could enter human food, not only through the direct vegetal–human way but also through a more complex progress through food chain: vegetal → animal feed → animal tissues and derived products → human consumer.

Origin and Toxicological Properties

Synthesis

The four natural aflatoxins (B1, B2, G1 and G2) can be produced by fungal species belonging to *Aspergillus* genus, mainly *Aspergillus flavus* and *Aspergillus parasiticus* (Klich & Pitt, 1968; Rapper & Fennel, 1965). These are worldwide common contaminants of wide variety of commodities and therefore aflatoxins may be found in many vegetal products, from cereals to groundnuts, cotton seeds, dry fruits, spices, etc. (Detroy, Lillehoj, & Ciegler, 1971; Diener et al., 1987; Fazekas, Tar, & Kovacs, 2005; Senyuva, Gilbert, & Ulken, 2007; Toteja et al., 2006; Zinedine et al., 2007). If these fungal species can grow and produce toxins in the field or during storage, climatic conditions required for their development were often associated with tropical areas (high humidity of the air, temperature ranging from 25 to 40°C) (Kaaya & Kyamuhangire, 2006; Northolt & Van Hegmond, 1981; Sanchis & Magan, 2004; Thompson & Henke, 2000; Trenk & Hartman, 1970). However, following extreme climatic conditions (abnormally hot summer period), aflatoxins could be found in other parts of the world. For example, in 2003, maize harvested in some parts of Europe was found contaminated by unusual AFB1 concentrations whereas European crops are usually considered as aflatoxin free (Battilani, 2005; Giorni, Magan, Pietri, Bertuzzi, & Battilani, 2007).

When present in vegetal matrix, aflatoxins are stable and weakly sensitive to thermal treatments (sterilization or freezing) or drying step (Hawkins, Windham, & Williams, 2005; Park, 2002). Many studies focused on possible detoxification process. Among them, for oilcakes intended for animal feed, ammoniac treatment can be performed to decrease aflatoxin concentration (Bailey, Price, Par, & Hendricks, 1982; Martinez, Weng, & Park, 1994; Park, 2002; Weng, Martinez, & Park, 1994).

Toxicity

Aflatoxin B1 is a highly carcinogenic agent leading to primary hepatocarcinoma (JECFA, 1999; Newberne & Butler, 1969; Peers & Linsell, 1973; Shank, Bhamarapravati, Gordon, & Wogan, 1972). This property is directly linked to its metabolism and to the appearance of the highly reactive epoxide derivative (see below). Formation of DNA adducts of AFB1-epoxide is well characterized (Cullen & Newberne, 1994). The primary site of adduct formation is the N7 position of the guanine nucleotide. Differences in AFB1 metabolism within animal species could explain the variability of the response in terms of carcinogenic potential of the mycotoxin (Eaton & Ramsdel, 1992; Gallagher & Eaton, 1995).

AFM1 can be considered as a genotoxic agent but its carcinogenic potential is weaker than that of AFB1 (JECFA, 2001). Taking into account the toxicity of these molecules, International Agency for Research against Cancer (IARC) classified AFB1 in the group 1 of carcinogenic agents, AFM1 in the 2B group of molecules that are carcinogenic in animals and possibly carcinogen in human and AFG1 in the group 3 of non-carcinogenic compounds (IARC, 1993).

Regulation

Due to their carcinogenic potential, JECFA did not define maximal tolerable daily intake for aflatoxins. Indeed, these molecules being cancer initiators, the most realistic way to protect consumers against these contaminants is to reduce human exposure to the "as low as reasonably achievable" level (Trischer, 2004).

In 2003, among the 99 countries that have implemented regulation for mycotoxins content in foods, all had at least regulatory value for aflatoxin B1 or total aflatoxins content (FAO, 2004). Most of these regulations concern vegetal raw material intended for human or animal consumption and milk, as illustrated by European Union regulation (Table 4.3) (European Union, 2001). However, in some particular countries such as Ukraine or Serbia, there is a specific regulation for meat and meat products whereas in many other countries, regulatory limits are applied to all foods intended to human consumption (FAO, 2004).

Contamination of Meat and Meat Products

Metabolism sulfo-conjugated forms of AFM1, AFQ1 and AFP1 (for review Guengerich et al., 1998)

Absorption of aflatoxin B1 administrated by oral route is rapid and almost complete (Gregory, Goldstein, & Edds, 1983). Absorption takes place in the

Table 4.3 EU regulation for aflatoxins contamination (μg/kg)

Destination	Toxin	Matrix	Maximal concentration (μg/kg)
Human food	Aflatoxin B1	Groundnuts + grains + dry fruits	2, 5 or 8 depending on the product and the processing step
		Cereals	2 or 5 depending on the product and the processing step
		Spices	5
		Cereal-based foods for young children	0.1
	Aflatoxins B1 + B2 + G1 + G2	Groundnuts + grains + dry fruits	4, 10 or 15 depending on the product and the processing step
		Cereals	4 or 10 depending on the product and the processing step
		Spices	10
	Aflatoxin M1	Milk	0.05
		Preparation for young children	0.025
Animal feed	Aflatoxin B1	Raw material for animal feeds	20
		Compound feeds	5–20 depending on animal species

small intestine, mainly in jejunum (Kumagai, 1989). In plasma, AFB1 is strongly linked to albumin, part of this fixation being covalent. Then, AFB1 goes through the liver where most part of the toxin is going to be metabolized, only 1–10% of the AFB1 staying fixed to macromolecules. AFB1 is a lipophilic molecule that will go through classical phase I and II biotransformation process. Main phase I metabolites are epoxide, hydroxyled compounds AFM1, AFQ1, AFP1, a reduced compound: aflatoxicol (AFL), a hydrogenated and hydroxylated molecule: AFB2a (Fig. 4.1). The most important phase II metabolites of the epoxide derivative are conjugated with GSH and glucorono- and sulfo-conjugated forms of AFM1, AFQ1 and AFP1.

Epoxide formation can be considered as a bioactivation process due to the very high reactivity of this molecule with liver macromolecules. Other metabolic pathways may be considered as detoxification leading to compounds without any toxicity (AFQ1, AFP1, AFB2a) or that keep residual toxicity (AFM1 and aflatoxicol).

Excretion of aflatoxins is quite slow (70–80% of a single dose in 4 days), biliary excretion being the most important route (50%) and AFM1 the major excreted metabolite.

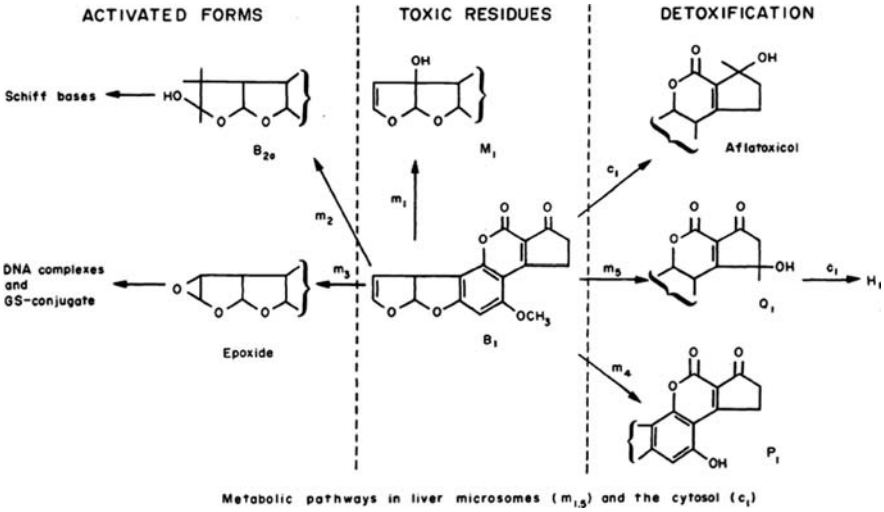

Fig. 4.1 Phase I metabolism of aflatoxin B1 (Paterson, 1977)

Residual Persistence in Meat

The very intense metabolism of AFB1 in the liver explains that only a very small part of the native molecule can be detected in animal tissues (Table 4.4). Whatever the study and the method used for quantification, liver and kidney always contain more toxin and metabolites than muscles (Beaver et al., 1990; Bintvihok, Thiengnin, Doi, & Kumagai, 2002; Miller et al, 1982; Stubblefield, Honstead, & Shotwell, 1991; Trucksess et al., 1982; Trucksess, Stoloff, Young, Wyatt, & Miller, 1983). In muscles, only low levels are found, often below detection limits of the methods used, even after exposure of the animals to high doses of aflatoxin B1 (Hirano, Adachi, Bintvihok, Ishibashi, & Kumazawa, 1992; Stubblefield et al., 1991; Trucksess et al., 1982). In ruminants, it has to be noted that many studies evaluate the transfer of aflatoxin in the milk of lactating cows (Battacone et al., 2003, 2005; Fremy & Quillardet, 1985; Frobish, Bradley, Wagner, Long-Bradley, & Hairston, 1986; Kiermeier, 1973; Veldman, Mejs, Borggreve, & van der Tol Heeres, 1992), whereas no complete data is available on the carryover of the molecule in muscle of cattle. However, as for other species, residues can be found in liver and kidney that are edible parts of these animals (Shreeve, Patterson, & Roberts, 1979; Stubblefield, Pier, Richard, & Shotwell, 1983).

Synthesis During Meat Processing

Several studies indicated that dry cured meats can be contaminated with toxigenic *A. flavus* strains, especially when products are processed in countries with hot climate (Aziz & Youssef, 1991; Cvetnik & Pepeljnjak, 1995; El Kady,

Table 4.4 Residues of aflatoxin in animal tissues (only the most demonstrative studies are reported)

Animal species	Dose and duration of exposure	Tissues	Residues (μg/kg)	Metabolites	References
Poultry					
Turkey	50 and 150 μg/kg feed for 11 weeks	Liver	0.02–0.009 and 0.11–0.23	AFB1 + AFM1	Paterson (1977)
		Kidney	0.02–0.04 and 0.11–0.21	AFB1 + AFM1	
		Gizzard	0.04–0.16 and 0.01–0.12	AFB1 (AFM1 < 0.01)	
Turkey	50 and 150 μg/kg feed for 11 weeks and 1 week with toxin-free feed	Liver	< 0.01	AFB1 + AFM1	
		Kidney	< 0.01	AFB1 + AFM1	
		Gizzard	0.04–1.9 and 0.09–0.24	AFB1 (AFM1 < 0.01)	
Quail	3,000 μg/kg feed for 8 days	Liver	7.83 ± 0.49 and 5.31 ± 0.22	Free and conjugated AFB1	Richard et al. (1986)
			22.34 ± 2.4 and 10.54 ± 0.42	Free and conjugated metabolites	
		Muscle	0.38 ± 0.03 and < 0.03	Free and conjugated AFB1	
			0.82 ± 0.05 and 0.32 ± 0.08	Free and conjugated metabolites	
Duck	3,000 μg/kg feed for 8 days	Liver	0.52 ± 0.04 and 0.44 ± 0.16	Free and conjugated AFB1	
			2.74 ± 0.15 and 3.81 ± 0.25	Free and conjugated metabolites	
		Muscle	< 0.03 and < 0.03	Free and conjugated AFB1	
			0.21 ± 0.09 and 0.14 ± 0.05	Free and conjugated metabolites	
Chicken	3,000 μg/kg feed for 8 days	Liver	0.15 ± 0.09 and 0.10 ± 0.01	Free and conjugated AFB1	
			1.54 ± 0.36 and 0.93 ± 0.04	Free and conjugated metabolites	
		Muscle	< 0.03 and < 0.03	Free and conjugated AFB1	
			0.11 ± 0.02 and 0.08 ± 0.05	Free and conjugated metabolites	
Hen	3,000 μg/kg feed for 8 days	Liver	0.34 ± 0.03 and 0.23 ± 0.08	Free and conjugated AFB1	
			2.38 ± 0.36 and 4.04 ± 0.1	Free and conjugated metabolites	
		Muscle	< 0.03 and < 0.03	Free and conjugated AFB1	
			0.14 ± 0.04 and 0.11 ± 0.04	Free and conjugated metabolites	
Laying hen	10,000 μg/kg feed for 7 days	Eggs	0.28 ± 0.1 and 0.38 ± 0.11	AFB1 and total aflatoxicol	Qureshi, Brake, Hamilton, Hagler, and Nesheim (1998)
Laying hen	8,000 μg/kg feed for 7 days	Liver	0.49 ± 0.28 and 0.2 ± 0.09	AFB1 and total aflatoxicol	Trucksess et al. (1983)
		Kidney	0.32 ± 0.18 and 0.1 ± 0.04	AFB1 and total aflatoxicol	

Table 4.4 (continued)

Animal species	Dose and duration of exposure	Tissues	Residues (μg/kg)	Metabolites	References
		Muscle	0.08 ± 0.03	Aflatoxicol	
		Eggs	0.24 ± 0.07 and 0.25 ± 0.09	AFB1 and total aflatoxicol	
Laying hen	2,500 μg/kg feed for 4 weeks	Liver	4.13 ± 1.95	AFB1	Zaghini, Martelli, Roncada, Simioli, and Rizzi (2005)
		Eggs	< 0.5 and < 0.01	AFB1 and AFM1	
Chicken	55 μg/kg feed for 9 days	Liver	0.26 and 0.02	AFB1 and AFM1	Madden and Stahr (1992)
Chicken	4,448 μg/kg feed for 9 days	Liver	1.52 and < 0.1	AFB1 and AFM1	
Swine					
Adult pigs	395 μg/kg feed for 14 days	Liver	1.242 ± 0.44	AFB1, AFM1 and aflatoxicol	Trucksess et al. (1982)
		Kidney	1.606 ± 0.63	AFB1, AFM1 and aflatoxicol	
		Muscle	0.16 ± 0.22	AFB1, AFM1 and aflatoxicol	
Growing mixed breed swine	400 μg/kg feed for 10 weeks	Liver	1.43	Total aflatoxins + AFM1	Miller et al. (1982)
		Kidney	0.83	Total aflatoxins + AFM1	
		Muscle	ND	Total aflatoxins + AFM1	
Growing mixed breed swine	800 μg/kg feed for 10 weeks	Liver	2.81	Total aflatoxins + AFM1	Miller et al. (1982)
		Kidney	1.21	Total aflatoxins + AFM1	
		Muscle	0.64	Total aflatoxins + AFM1	
Male miniature swine	590 μg/kg feed for 15 days	Liver	3.17	AFB1 + AFB2 + AFM1	Beaver et al. (1990)
		Kidney	6.47	AFB1 + AFB2 + AFM1	
		Muscle	0.52	AFB1 + AFB2 + AFM1	
Cross-breed pigs	524 μg/kg feed for 35 days	Liver	2.01	AFB1 + AFB2 + AFM1	Beaver et al. (1990)
		Kidney	3.951	AFB1 + AFB2 + AFM1	
		Muscle	0.443	AFB1 + AFB2 + AFM1	

El Maraghy, & Zorhi, 1994; Ismail & Zaky, 1999; Rojas, Jodral, Gosalvez, & Pozo, 1991). Moreover, it has been demonstrated that the processing conditions during ageing of hams may allow aflatoxin synthesis (Bullerman, Hartman, & Ayres, 1969). Therefore, it is of public health importance to evaluate the possible production of aflatoxin B1 during meat processing and ageing. Few studies were carried out but they all demonstrated that the frequency of contamination of processed meat with aflatoxin B1 was low and that the level of toxin within meat was usually below 10 ng/g (Aziz & Youssef, 1991; Ismail & Zaky, 1999). However, it is not clear weather aflatoxin B1 was produced during meat processing or was present before at the residual level in muscles. Indeed, it seems that there is no relationship between the presence of toxigenic strains of *A. flavus* and aflatoxin contamination of meat samples (Ismail & Zaky, 1999). The frequent contamination of spices and additives used in such meat processing may also represent a source of mycotoxin (Aziz & Youssef, 1991; Refai, Niazi, Aziz, & Khafaga, 2003). Moreover, it has been demonstrated that the use of spices contaminated with toxigenic mould strains as ingredient in sausage making may lead to a secondary contamination of the final product with aflatoxins (Aziz & Youssef, 1991; Guergue & Ramirez, 1977).

Ochratoxin A

Introduction

Ochratoxins A, B and C are secondary metabolites produced by several *Aspergillus* and *Penicillium* species. According to its prevalence and toxicity, only ochratoxin A (OTA) will be treated in this part. This toxin has been found as a contaminant of many foodstuffs, mainly cereals but also wine and coffee. Meat and meat products may also contain OTA if animals are exposed to contaminated feed. Therefore, many studies have tried to characterize the importance of meat as a source of human exposure to this contaminant.

Origin and Toxicological Properties

Synthesis

Ochratoxin A can be produced by *Aspergillus ochraceus* (Van der Merwe, Steyn, Fourie, Scott, & Theron, 1965), *A. carbonarius* (Abarca, Accensi, Bragulat, Castella, & Cabanes, 2003; Belli, Ramos, Coronas, Sanchis, & Marin, 2005), *A. alliaceus* (Bayman, Baker, Doster, Michailides, & Mahoney, 2002) and *A. niger* (Abarca, Bragulat, Castella, & Cabanes, 1994) although the frequency of toxigenic strains in this species appears moderate (Hajjaji et al., 2006; Romero et al., 2005; Teren, Varga, Hamari, Rinyu, & Kevei, 1996). OTA can also be synthesized by *Penicillium* species, mainly *P. verrucosum* (previously

named *P. virridicatum*) (Pardo, Marin, Ramos, & Sanchis, 2006; Pitt, 1987). The ability of both *Aspergillus* and *Penicillium* species to produce OTA makes it a worldwide contaminant of numerous foodstuffs. Indeed, *Aspergillus* is usually found in tropical or subtropical regions whereas *Penicillium* is a very common contaminant in temperate and cold climate areas (Magan & Aldred, 2005; Pardo, Marin, Ramos, & Sanchis, 2005; Pardo, Marin, Sanchis, & Ramos, 2004; Pitt & Hocking, 1977). Many surveys revealed the contamination of large variety of vegetal products such as cereals (Jorgensen, 2005; Sangare-Tigori et al., 2006), grapes (Battilani, Giorni, Bertuzzi, Formenti, & Pietri, 2006; Battilani, Magan, & Logrieco, 2006) and coffee (Jorgensen, 2005; Taniwaki, 2006). For cereals, OTA contamination generally occurs during storage, especially when moisture and temperature are abnormally high whereas for coffee and wine, contamination occurs in the field or during the drying step (Bucheli & Taniwaki, 2002; Cairns-Fuller, Aldred, & Magan, 2005; MacDonald, Prickett, Wildey, & Chan, 2004; Magan & Aldred, 2005). When ingested by animals, OTA can be found at residue level in several edible organs (see below). Therefore, the consumption of meat contaminated with OTA has also been suspected to represent a source of exposure for humans (JECFA, 2001).

Toxicity

Kidney is the primary target of OTA. This molecule is nephrotoxic in all animal species. For example, OTA is considered as responsible for a porcine nephropathy that has been studied intensively in the Scandinavian countries (Elling, 1983; Krogh, 1977). This disease is endemic in Denmark where rates of porcine nephropathy and ochratoxin contamination of pig feed are highly correlated (Krogh, 1991). Because the renal lesions observed in pig kidneys after exposure to OTA are quite similar to that observed in kidneys of patients suffering from Balkan endemic nephropathy (BEN), OTA is suspected to play a role in this human syndrome (Castegnaro et al., 2006; Fuchs & Peraica, 2005; Plestina et al., 1982). BEN is a progressive chronic nephropathy that occurs in populations living in areas bordering Danube River in Romania, Bulgaria, Serbia and Croatia (Abouzied et al., 2002; Vrabcheva et al., 2004).

OTA disturbs cellular physiology in multiple ways. The primary effect could be associated with the enzymes involved in the phenylalanine metabolism, mostly by inhibiting the synthesis of the phenylalanine tRNA complex (Marquardt & Frolich, 1992). Moreover, OTA inhibits mitochondrial ATP production (Meisner & Meisner, 1981) and stimulates lipid peroxydation (Rahimtula, Bereziat, Bussacchini-Griot, & Bartsch, 1988).

In addition to its nephrotoxic effect, ochratoxin A appears to be a potent teratogen and a carcinogenic agent in animals (Bendele, Carlton, Krogh, & Lillehoj, 1985) and therefore has been classified by the IARC in the 2B group of molecules that are carcinogenic to animals and potentially carcinogen in humans (IARC, 1993). This property could be related to the effect of OTA on DNA, leading to the appearance of DNA breakage (Creppy et al., 1985) and

adducts (Obrecht-Pflumio & Dirheimer, 2001; Pfohl-Leszkowicz, Chakor, Creppy, & Dirheimer, 1991; Pfohl-Leszkowicz, Grosse, Kane, Creppy, & Dirheimer, 1993).

Regulation

Several maximal tolerable doses were determined for OTA. The first one, proposed by JECFA (JECFA, 1990), corresponds to a daily dose of 16 ng/kg body weight. It has been calculated taking into account the renal toxicity of OTA in pigs after a sub-chronic toxicity study.

By contrast, European Scientific Committee on human nutrition and the French High Committee of Public Hygiene (CSHPF) proposed a tolerable daily dose of 5 ng/kg body weight, taking into account the carcinogenic effects observed in rats (CSHPF, 1999).

These doses were used to build regulatory values in different foods and feeds (Table 4.5). In most cases, these values are of few ng/g. Some few countries included specific regulation for meat and meat products. For example, Denmark has a specific regulation for OTA content in pig kidneys and Italy has one for pig meat and derived products.

Table 4.5 Maximum level of OTA in some food and feed as regulated in different countries (FAO, 2004)

Commodity	Level (ng/g)	Country
Raw cereal grains	5	EU
All cereal-derived products	3	EU
	50	Israel
	2	Switzerland
Dried vine fruits	10	EU
Children foods	6	Czech Republic
Infant foods	1	
Pork meat and derived products	1[a]	Italy
Pig kidneys	10[b]	Denmark
	25[c]	
Coffee	5[a]	Finland
Roasted and instant coffee	4[a]	Italy
Raw coffee beans	20	Greece
Cocoa-derived products	0.5[a]	Italy
Grains for feed	300	Romania
Foods (all)	5	
Foodstuffs for poultry	200	Sweden
Foodstuffs for pigs	100	
Rice, barley, beans, coffee, maize	50	Uruguay

[a] guideline values;
[b] viscera condemned;
[c] whole carcass condemned

Contamination of Meat and Meat Products

Metabolism (for review, see Ringot, Chango, Schneider, & Larondelle, 2006)

OTA is partly absorbed at the stomach level but main absorption takes place in the small intestine (Kumagai & Aibara, 1982). Ochratoxin A is hydrolysed to a non toxic derivative of OTA by both carbopeptidase and chymotripsin but also by microbial flora of the digestive tract (Galtier & Alvinerie, 1976; Höhler, Sudekum, Wolffram, Frolich, & Marquardt, 1999; Hult, Teiling, & Gatenberg, 1976). In ruminants, this degradation takes place before the absorption of the toxin. That considerably limits the risk of having OTA contamination of meat and milk but metabolites can be detected in these tissues (Boudra, Barnouin, Dragaccci, & Morgavi, 2007; Valenta & Goll, 1996).

At the hepatic level, OTA is detoxified in several minor metabolites such as 4-hydroxy-ochratoxin.

The very strong affinity of the toxin for plasmatic proteins may slow down the elimination (Galtier & Alvinerie, 1976). Big differences have been reported for OTA half-life depending on animal species. Humans appear to display the longest half-life (more than 30 days) (Creppy, 1999).

Tissues distribution of the toxin revealed that the toxin concentration was, in decreasing order, as following: kidney > liver > muscle > fat (Table 4.6).

The re-absorption of OTA in kidney tubules via anionic transporters favours its renal accumulation.

Residual Persistence

Recent surveys done in European countries demonstrated that the role of meat products in human exposure to OTA can be considered as low (Leblanc, 2004). Indeed, meat and meat products only represent 3% of the OTA source in human diet (Jorgensen, 2005). The prevalence of contamination appears to be more important in northern Europe (SCOOP Report, 2000).

Synthesis During Meat Processing

The presence of toxigenic fungal strains on dry cured meat products (Escher, Koehler, & Ayers, 1973; Tabuc et al., 2004) raises the possibility of the direct contamination of these foods. Many surveys were conducted to evaluate contamination of dry meat products. However, these surveys essentially demonstrated the possible carryover of the ochratoxin A in processed meat. Indeed, even if ochratoxigenic moulds have been isolated from such foods (Battilani et al., 2007; Bogs, Battilani, & Geisen, 2006; Tabuc et al., 2004), it appears that ripening and ageing conditions are not favourable to toxin production and the production of OTA on meat products after contamination with toxigenic strains seems to remain quite low, even if this mycotoxin appears to be stable in meat products (Bailly et al., 2005; Escher et al., 1973).

Table 4.6 Occurrence of OTA in meat products from different countries

Sample (country)	Number of sample analysed	% positive (level ng/g)	% of samples with level > 1 ng/g	Maximum observed level (ng/g)	References
Pork meat (DK)	76	84.2 (> 0.02)	2.6	1.3	Hult et al., 1979
Pork kidney (DK)	300	94.6 (> 0.02)	8	15	Jorgensen, 1998
Pork kidney (F)	300	1 (> 0.4)		1.4	Dragacci et al., 1999
	710	7.6 (> 0.5)		5	
Pork meat (DK)	300	76 (> 0.02)	3	2.9	Dragacci et al., 1998
Ham (I)	42	100 (> 0.04)	35.7	2.3	Jorgensen, 1998
Pork meat products (I)	106	23 (> 0.03)		28.4	Terada et al., 1986
Pork edible offals (D)	102	69 (0.01)	0.03	9.33	
Sausage (D)	201	52 (> 0.01)	0.01	4.56	Pietri et al., 2006
Ham (D)	57	72 (> 0.01)	0	0.17	SCOOP report, 2000
					SCOOP report, 2000
					SCOOP report, 2000

Zearalenone

Introduction

Zearalenone (ZEA) is a mycotoxin with estrogenic effect that is produced by *Fusarium* species. Recently, endocrine disrupters received a lot of public attention since they are suspected to reduce male fertility in human and wildlife populations and possibly involved in cancer development (Stopper, Schmitt, & Kobras, 2005). This molecule is well known to farmers, often responsible for reproduction perturbation, especially in pigs. Therefore, ZEA content is regulated in many foodstuffs and the carryover of this molecule in animal tissues has been investigated.

Origin and Toxicological Properties

Synthesis

Zearalenone has been isolated for the first time from maize contaminated with *Gibberella zeae*, the anamorph of *Fusarium graminearum* (Stob, Baldwin, Tuite, Andrews, & Gilette, 1962). It has been demonstrated that it can be synthesized by several *Fusarium* species such as *F. graminearum, F. proliferatum, F. culmorum* and *F. oxysporum* (Molto, Gonzalez, Resnik, & Pereyra Gonzalez, 1997; Sydenham, Marasas, Thiel, Shephard, & Nieuwenhuis, 1991). These are fungal species that usually develop on living plants and ZEA contamination occurs in the field, at harvest or early storage, when the drying step was not sufficient. Indeed, *Fusarium* growth and mycotoxin production usually occur at high water activity (> 0.90) (Jimenez, Manez, & Hernandez, 1996; Montani, Vaamonde, Resnik, & Buera, 1988). Temperature of ZEA production is lower than optimal temperature for mycelium development and is about 20–25°C (Llorens, 2004; Milano & Lopez, 1991; Ryu & Bullerman, 1999). Moreover, ZEA production is favoured in substrates with high glucid/protein ratio. Due to these parameters, ZEA is a frequent contaminant of cereals and cereal-derived products in European and other countries with temperate climate (Schothorst & Van Hegmond, 2004; Zinedine, Soriano, Molto, & Manes, 2007).

Toxicity

Acute toxicity of ZEA is usually considered as weak with LD50 after oral ingestion ranging from 2,000 to more than 20,000 mg/kg b.w. (JECFA, 2000; Kuiper-Goodman, Scott, & Watanabe, 1987). Sub-acute or chronic toxicity of the mycotoxin is more frequent and may be observed at the natural contamination levels of feeds. The effects are directly related to the fixation of ZEA and metabolites on estrogenic receptors. Affinity with estrogenic receptors is, in decreasing order, α-zearalanol > α-zearalenol > β-zearalanol > ZEA > β-zearalenol. Pig and sheep appear more sensitive than other animal species:

in multiple exposure experiments, the non-observed effect level (NOEL) in pigs was 40 μg/kg of body weight whereas it was 100 μg/kg b.w. in rats (JECFA, 2000; Kuiper-Goodman et al., 1987).

ZEA induces alteration in the reproductive tracts of both laboratory and farm animals. Variable estrogenic effects have been described, such as a decrease of the fertility, a decrease of litter size, an increase in embryo-lethal resorptions, change in adrenal, thyroid and pituitary glands weights. In male pigs, ZEA can depress testosterone, weight of testes and spermatogenesis while inducing feminization and suppressing libido (JECFA, 2000; Kuiper-Goodman et al., 1987; Zinedine, Soriano, Molto, & Manes, 2007). No teratogenic effect was observed in laboratory animals.

Studies reported that several alterations in immunological parameters could be observed in vitro after ZEA exposure of mice or human cells (Berek, Petri, Mesterhazy, Teren, & Molnar, 2001; Marin, Murtha, Dong, & Pestka, 1996).

Long-term exposure studies did not allow the demonstration of any carcinogenic potential of this mycotoxin. Therefore, zearalenone has been classified by IARC as an estrogenic molecule in the group III of non-carcinogenic molecules (IARC, 1993).

Regulation

In 1999, JECFA established a temporary maximal daily tolerable dose of 0.5 μg/kg body weight. It is based on the hormonal effects observed in the most sensitive species (pigs) and the NOEL of 50 μg/kg b.w./day with a security factor of 100 (JECFA, 2000). In France, the CSPHF proposed a daily tolerable dose of 0.1 μg/kg b.w./day calculated on effects observed in monkey's reproduction (CSHPF, 1999).

In 2003, ZEA was regulated in foods and feeds by 16 countries and in 2006 and 2007, EU adopted regulation and recommendation for ZEA in human foods and animal feeds, respectively (European Union, 2006, 2007).

Contamination of Meat and Meat Products

Metabolism

ZEA is quickly absorbed after oral ingestion (Dailey, Reese, & Brouwer, 1980; Olsen, Malmlof, Pettersson, Sandholm, & Kiessling, 1985). Although no quantification has been reported, urinary excretion of zearalenone and its metabolites suggest that the absorption rate is high (Kuiper-Goodman et al., 1987; Mirocha, Pathre, & Robinson, 1981). For example, the uptake in a pig after a single oral dose of 10 mg/kg b.w. was estimated to be 80–85% (Biehl et al., 1993). Zearalenone can be metabolized in digestive tracts by both microflora and intestinal mucosa (Kollarczik, Garels, & Hanelt, 1994). This metabolism results in the appearance of α- and β-zearalenol and α- and β-zearalanol. The proportion of these two metabolites may change depending on the animal

species (Kallela & Vasenius, 1982; Olsen, Petersson, Sandholm, Visconti, & Kiessling, 1987; Zinedine, Soriano, Molto, & Manes, 2007). Because among zearalenone metabolites, α-zearalenol has a higher affinity for estrogenic receptors, its appearance during metabolic pathways can be considered as a bioactivation. Therefore, metabolism is a key factor of zearalenone toxicity and differences in toxin transformation within organism can explain differences in toxicity observed in several animal species (Gaumy, Bailly, Benard, & Guerre, 2001).

After absorption, two major hepatic biotransformation pathways have been suggested for zearalenone in animals (Kiessling & Pettersson, 1978; Olsen, Pettersson, & Kiessling, 1981):

- hydroxylation resulting in the formation of α-zearalenol and β-zearalenol
- conjugation of zearalenone and reduced metabolites with glucuronic acid

Differences between species in hepatic biotransformation have been demonstrated; pigs seem to convert zearalenone predominantly in α-zearalenol, whereas β-zearalenol is the main metabolite in cattle (Malekinejad, Colenbrander, & Fink-Gremmels, 2006). In human, as in pigs, zearalenone was found mainly as glucoronide conjugates of zearalenone and α-zearalenol in urine. All of the metabolites found in humans during the 24 h of sampling were glucuronides (Mirocha et al., 1981).

Zearalenone and metabolites are excreted in urine or bile (JECFA, 2000). In ruminants, ZEA and metabolites are detected in bile at respective rates of 68% β-zearalenol, 24% α-zearalenol and 8% zearalenone (Dänicke, Gadeken, Ueberschar, Meyer, & Scholz, 2002). In this study, neither zearalenone nor its metabolites were detected in muscles, kidney, liver or dorsal fat of bovine receiving 0.1 mg ZEA/day/kg feed.

Residual Contamination of Meat

Only few studies are available on the potential carryover of this mycotoxin in animal edible organs. It appears that, at least in pigs, meat and other edible parts may not be contaminated, even after exposure of the animals to high concentrations of the toxin (Baldwin, Williams, & Terry, 1983; Goyarts, Danicke, Valenta, & Ueberschaar, 2007; Sundlof & Strickland, 1986). In poultry, few studies done with very high doses of zearalenone allowed the detection of the toxin at detectable level in muscles (Mirocha, Robison, Pawlosky, & Allen, 1982). More recently, an experiment of long-term exposure of laying hens with 1.58 mg ZEA/kg feed for 16 weeks did not allow the detection of any residues in muscles, fat or eggs (Dänicke et al., 2002).

Synthesis During Meat Processing

Production of zearalenone, as well as other fusariotoxins, cannot be observed in processed meats due to environmental conditions required for *Fusarium* development and toxinogenesis (mainly water activity) (Miller, 2002).

Trichothecenes

Introduction

Tricothecenes constitute a large group of secondary metabolites produced by numerous species of *Fusarium*, such as *F. graminearum, F. culmorum, F. poae* and *F. sporotrichioides*. More than 160 trichothecenes have been identified, notably deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), fusarenon X. DON is the most frequently found trichothecene. These mycotoxins are important for public health concern; however, due to their metabolism pathways in animals, they do not represent a significant hazard as residual contaminant of muscle foods.

Origin and Toxicological Properties

Synthesis

Trichothecenes can be produced by a large variety of fungus (Table 4.7). They mainly belong to the *Fusarium* genus; however, other fungal species such as *Trichoderma viridae* or *Myrothecium roridum* have also been shown able to produce some trichothecenes (Bean, Jarvis, & Aboul-Nasr, 1992; Wilkins, Nielsen, & Din, 2003). One fungal species may be able to produce several trichothecenes.

These fungal species mainly belong to the field mycoflora, developing on living plants or during the early post-harvest period. Indeed, *Fusarium* species are hygrophilic fungus and the drying step will block their development (Schrödter, 2004). These fungal contaminants are well known for being responsible for *Fusarium* head blight of small grain cereals and ear rot of maize. These fungal pathologies reduce yields, decrease milling and malting qualities of grains and may lead to mycotoxin contamination of infected grains (Logrieco, Mule, Moretti, & Bottalico, 2002; Parry, Jenkinson, & McLeod, 1995). As for all mycotoxin production, climatic conditions directly influence the trichothecene synthesis. Moreover, fungal development and subsequent trichothecene production may be related to agricultural practices such as crop rotation (Edwards, 2004). For example, it is generally accepted that wheat that follows an alternative host for *Fusarium* pathogen (i.e. maize) is at greater risk for DON contamination of grain (Obst, Lepschy, Beck, Bauer, & Bechtel, 2000).

Due to their synthesis conditions, trichothecenes are frequent worldwide contaminants of cereals, mainly wheat and maize, and cereal-based products (F. Q. Li, Y. W. Li, Luo, & Yoshizawa, 2002; Pan, Bonsignore, Rivas, Perera, & Bettucci, 2007; Schothorst & Van Eegmond, 2004; Tanaka et al., 1988; Trucksess et al., 1996). The conditions during malting of grains may allow *Fusarium* development and trichothecene production. DON has been found as a frequent contaminant of beers, even if contamination levels are usually low (Molto,

Table 4.7 Main fungal species able to produce trichothecenes

Fungal genus	Fungal species	Toxins	Type
Fusarium	*Fusarium tricinctum*	DAS, T-2 toxin, HT-2 toxin	A
	Fusarium sporotrichoides	DAS, T-2 toxin, HT-2 toxin	
	Fusarium poae	DAS, T-2 toxin, HT-2 toxin, acetyl T-2 toxin	
	Fusarium solani	DAS, T-2 toxin, HT-2 toxin	
	Fusarium roseum	Scirpenol, monoacetoxyscirpenol	
	Fusarium nivale	DON, NIV, fusarenon X	B
	Fusarium crookwellense	DON, fusarenon X	
	Fusarium oxysporum	DON, diacetyl-NIV, fusarenon X	
	Fusarium avanaceum	DON, fusarenon X	
	Fusarium graminearum	DON, fusarenon X	
	Fusarium solani	DAS, T-2 toxin, HT-2 toxin, DON, fusarenon X	A and B
	Fusarium culmorum	DAS, T-2 toxin, HT-2 toxin, diacetyl-NIV	
	Fusarium equiseti	DAS, T-2 toxin, HT-2 toxin, diacetyl-NIV, fusarenon X	
Trichoderma	*Trichoderma viridae*	Trichodermin	A
	Trichoderma polysporum	Trichodermin, roridin C	
	Trichoderma lignorum	Trichodermin, T-2 toxin	
Myrothecium	*Myrothecium roridum*	Trichodermadienediol, roridin C	A
Trichothecium	*Trichothecium roseum*	Trichothecin	B

Samar, Resnik, Martinez, & Pacin, 2000; Papadopoulou-Bouraoui, Vrabcheva, Valzacchi, Stroka, & Anklam, 2004; Wolf-Hall & Schwarz, 2002).

Toxicity

Because trichothecenes are a large family grouping many compounds of variable structure and properties, their toxicity can be very different depending on the molecule, the animal species, the dose and the exposure period. There are many reviews available on trichothecenes toxicity (Pestka & Smolinski, 2005; Pieters et al., 2002; Rocha, Ansari, & Doohan, 2005; Rotter, Prelusky, & Petska, 1996) and only the mains features will be presented here.

Trichothecenes are potent inhibitors of eukaryotic protein synthesis, interfering with initiation, elongation or termination stages.

Concerning their toxicity in animals, DAS, DON and T-2 toxin are the most studied molecules. The symptoms include effects on almost all major systems of organism; many of them being secondary processed initiated by poorly understood metabolic process in relation with protein synthesis inhibition.

Among naturally occurring trichothecenes, DAS and T-2 toxin seem to be the most potent in animal. They have an immunosuppressive effect, decreasing resistance to microbial infections (Rotter et al., 1996). They also cause a wide range of gastrointestinal, dermatological and neurological symptoms (Trenholm, Friend, Hamilton, Prelusky, & Foster, 1989). In human, these molecules have been suspected to be associated with alimentary toxic aleukia. The disease, often reported in Russia during the nineteenth century, is characterized by inflammation of the skin, vomiting, damage to haematopoietic tissues (Joffe, 1978; Lutsky, Mor, Yagen, & Joffe, 1978).

When ingested at high concentrations, DON causes nausea, vomiting and diarrhoea. At lower doses, pigs and other farm animal display weight loss and food refusal (Rotter et al., 1996). For this reason, DON is often called vomitoxin or food refusal factor.

Regulation

In 1993, IARC classified trichothecenes (T-2 toxin, DON and NIV) in the group 3 of compounds with inadequate data in human and animals to rule on their carcinogenicity (IARC, 1993).

However, several daily tolerable doses were fixed by JECFA, according to toxic effects observed in pigs or rodents. These doses are 0.06, 1 and 0.7 μg/kg b.w./day for T-2, DON and NIV, respectively.

Several countries have implemented a regulation for trichothecenes content in food and feed. These regulatory values or recommendations mainly concern DON and are active in countries where climatic conditions are suitable for *Fusarium* development and toxinogenesis (Europe and northern countries).

Contamination of Meat and Meat Products

Metabolism

Absorption kinetics have not been reported for all trichothecenes; however, they seem to be rapidly and efficiently absorbed in gastrointestinal tract, whatever the animal species (Cavret & Lecoeur, 2005). DON, NIV or T-2 toxin can be detected in blood less than 30 min after ingestion (Eriksen & Petterson, 2004). Absorption rates range from 10 to 55%. Trichothecenes can be metabolized within the digestive tracts by ruminal flora. It may partially explain the ruminant resistance to these toxic compounds (Yiannikouris & Jouany, 2002). In other species, intestinal microflora may also metabolize trichothecene (Young, Zhou, Yu, Zhu, & Gong, 2007). DON transformation in digestive tract is well documented (He, Young, & Forsberg, 1992; Swanson, Helaszek, Buck, Rood, & Haschek, 1988). Few traces of de-epoxy-DON are found in pig stomach and small intestine whereas its quantity increases in large intestine to reach 80% of the toxin in rectum (Dänicke, Valenta, & Döll, 2004).

After absorption, metabolism of trichothecenes mainly consists in hydrolysis, hydroxylation and de-epoxydation. This metabolism differs according to animal species but it generally leads to the reduction of the epoxy group of the molecules and glucuronidation (Cavret & Lecoeur, 2005; Yiannikouris & Jouany, 2002). For example, in pigs, more than 95% of the ingested dose of DON is excreted without any transformation and less than 5% is found as its glucuronide metabolite (Prelusky, Hartin, Trenholm, & Miller, 1988).

When absorbed, trichothecenes are rapidly eliminated without any accumulation in the organism (Eriksen & Petterson, 2004). Only traces of compounds can be still detected 24 h after ingestion (Dänicke et al., 2004; Dänicke, Valenta, Ueberschar, & Matthes, 2007; Prelusky et al., 1988). Plasmatic half-lives of trichothecenes are of several hours (Cavret & Lecoeur, 2005).

Residual Contamination of Meat

The available data on metabolism explain that meat and meat products are not considered as a potential source of trichothecenes for human consumers. Indeed, the starvation diet that always precedes animal slaughtering is usually long enough to allow trichothecene elimination from edible parts of the organism (Coppock et al., 1985; Prelusky et al, 1988). In case of animal exposure till slaughtering, trichothecenes are only found as traces, even after repeated exposure in pigs (Pollmann, Koch, Seitz, Mohr, & Kennedy, 1985; Prelusky & Trenholm, 1992) as well as in poultry (Prelusky, Hamilton, Trenholm, & Miller, 1986; Chi, Robison, Mirocha, Behrens, & Shimoda, 1978; El-Banna, Hamilton, & Scott, & Trenholm, 1983).

Therefore, all studies on the carryover of trichothecenes in edible parts of exposed animals suggest that meat and meat products cannot be considered as a source of exposure to these toxins.

However, only few methods were developed for trichothecenes analysis in animal tissues. Indeed, experiments on the pharmacokinetics and distribution of these mycotoxins were firstly performed using radio-labelled toxins and revealed that trichothecenes were rapidly excreted and carryover of the toxins in edible part of animals was minimal (Chi et al., 1978; Giroir, Ivie, & Huff, 1991; Prelusky et al., 1986, 1988; Yoshizawa, Swanson, & Mirocha, 1980).

Synthesis During Meat Processing

As explained for zearalenone, trichothecene production cannot be observed on processed meats due to environmental conditions required for *Fusarium* development and toxinogenesis (Miller, 2002).

Fumonisins

Introduction

Even if their effects on animal health, especially equines, were known for long time, fumonisins have been identified in 1988. From this period, this family of mycotoxins has been extensively studied and revealed several characteristics concerning physico-chemical properties, metabolism and mechanism of action. If these toxins appear to be of great importance for human and animal health concern, it has also been demonstrated that, due to their absorption and kinetic properties, meat and meat products should not represent an important source of exposure of human consumers. This part will be focused on fumonisin B1 (FB1), the most abundant and toxic compound of this family.

Origin and Toxicological Properties

Synthesis

Fumonisins were first described and characterized in 1988 from *Fusarium verticillioides* (formerly *F. moniliforme*) culture material (Gelderblom et al., 1988). The most abundant and toxic member of the family is fumonisin B1. These molecules can be produced by few species of *Fusarium* fungi: *F. verticillioides, F. proliferatum* and *F. nygamai* (Marin, Magan, Ramos, & Sanchis, 2004; Rheeder, Prasanna, & Vismer, 2002). These fungal species are worldwide contaminants of maize that represent the main source of fumonisins. *F. verticillioides* grows as an endophyte in corn, and even if it can cause plant pathology such as seedling blight, stalk rot and ear rot (Nelson, Desjardin, & Plattner, 1993), fumonisin contamination of grains can occur without any visible alteration.

Fumonisin production mainly occurs during peri-harvest period, at temperatures about 20–25°C and high moisture content of grains (J. Le Bars, P. Le Bars, Dupuy, & Boudra, 1994; Marin et al., 2004). Many surveys revealed that fumonisins are very major contaminants of maize and maize products worldwide (Afolabi, Bandyopadhyay, Leslie, & Ekpo, 2006; Curtui, Usleber, Dietrich, Lepschy, & Martlbauer, 1998; Kim, Shon, Chung, & Kim, 2002; Leblanc, Tard, Volatier, & Verger, 2005; SCOOP report, 2003; Shephard et al., 2002; Silva, Lino, Pena, & Molto, 2007; Sugita-Konishi et al., 2006).

Toxicity

One major characteristic of fumonisins is that they induce very different syndromes depending on the animal species. FB1 is responsible for equine leukoencephalomalacia characterized by necrosis and liquefaction of cerebral tissues (Bailly et al., 1996; Marasas et al., 1988). Horses appear to be the most sensitive species since clinical signs may appear after exposure to doses as low as 5 mg FB1/kg feed during few weeks. Pigs are also sensitive to FB1 toxicity. In this species, fumonisins induce pulmonary oedema after exposure to high doses (higher than 20 mg FB1/kg feed) of mycotoxins and hepatotoxic and immunotoxic at lower doses (Harrison, Colvin, , Greene, , Newman, & Cole, 1990; Harvey et al., 1996; Oswald et al., 2003). By contrast poultry and ruminants are more resistant to this mycotoxin and clinical signs only appear after exposure to doses higher than 100 mg FB1/kg which may be encountered in natural condition but are quite rare (Bailly, Benard, Jouglar, Durand, & Guerre, 2001; Bermudez, Ledoux, & Rottinghaus, 1995; Brown, Rottinghaus, & Williams, 1992; Diaz, Hopkins, Leonard, Hagler, & Whitlow, 2000; Ledoux, Brown, Weibking, & Rottinghaus, 1992; Osweiller et al., 1993). In rodents, FB1 is hepatotoxic and carcinogenic, leading to appearance of hepatocarcinoma in long-term feeding studies (Gelderblom, Semple, Marasas, & Farber, 1992; Gelderblom et al., 2001). In human, FB1 exposure has been correlated with high prevalence of oesophageal cancer in some parts of the world, mainly South Africa, China and Italy (Marasas, 1995). Finally, fumonisins can cause neural tube defects in experimental animals and thus may also have a role in human cases (Hendricks, 1999; Hendricks, Simpson, & Larsen, 1999; Marasas et al., 2004; Missmer, Suarez, Felkner, & Wang, 2006). At the cellular level, FB1 interacts with sphingolipid metabolism by inhibiting ceramide synthase (Merrill, Sullards, Wang, Voss, & Riley, 2001). This leads to the accumulation of free sphinganine (Sa) and, to a lesser extent, of free sphingosine (So). Therefore, the determination of the Sa/So ratio has been proposed as a biomarker of fumonisin exposure in all species where it has been studied (Garren, Galendo, Wild, & Castegnaro, 2001; Goel, Schumacher, Lenz, & Kemppainen, 1996; Tran et al., 2003; Van der Westhuizen, Shephard, & Van Schalkwyk, 2001). The accumulation of these active second messengers may perturb many cell function and lead to apoptosis, cell proliferation, membrane integrity disturbance, etc. (Desai et al., 2002; Riley et al., 1996; Strum, Ghosh, & Bell, 1997).

Due to its carcinogenic properties in laboratory animals, FB1 has been classified by IARC in the group 2B of molecules carcinogenic for animals and possibly carcinogenic in human (IARC, 1993).

Regulation

The European regulation set up maximal concentrations for fumonisin B1 and B2 in maize and maize-derived products for human food (European Union, 2007). Maximal limits are of 200–4,000 μg/kg depending on the food. EU also recommends the respect of maximal values in animal feeds. These values may change depending on the animal species and its sensitivity to the toxins. They range from 5 mg/kg for horses and pigs to 50 mg/kg for adult ruminants. Only few other countries such as the United States or Iran have regulatory limits for fumonisins in maize and maize products (FAO, 2004).

Contamination of Meat and Meat Products

Metabolism

After oral ingestion, fumonisin B1 is only weakly absorbed (1–5%) in all studied animal species (Prelusky et al., 1996; Prelusky, Savard, & Trenholm, 1995; Prelusky, Trenholm, & Savard, 1994; Vudathala, Prelusky, Ayroud, Trenholm, & Miller, 1994). More than 95% of the toxin is found as native form in faeces of exposed animals. Although FB1 is distributed in all tissues, most part of the absorbed toxin is found in liver and kidney (Martinez-Larranaga et al., 1999; Norred, Plattner, & Chamberlain, 1993). The toxin is not excreted in milk (Becker et al., 1995; Richard et al., 1996; Spotti et al., 2001). Both in vitro and in vivo experiments failed to demonstrate any metabolism of fumonisins (Cawood, Gelderblom, Alberts, & Snyman, 1994; Spotti, Pompa, & Caloni, 2001).

FB1 is excreted in faeces, bile and urine as native molecule or partially hydrolysed form (Meyer, Mohr, Bauer, Horn, & Kovacs, 2003; Norred et al., 1993; Shephard et al., 1994). Studies in weaned piglets revealed the presence of PHB1 and AP in tissue but confirmed that unmetabolized FB1 was the most abundant (Fodor et al., 2008).

Residual Contamination of Meat

Taking into account the toxico-kinetics parameters observed for FB1, it appears that edible parts of animals, and especially muscles, should not represent a source for human exposure. This was confirmed in swine after exposure of the animal to high dose of FB1 (Meyer et al., 2003). However, in some particular species such as ducks, taking into account the high proportion of

maize in diet (up to 99%), it may be important to evaluate the possibility of having FB1 residues in liver and muscles of animals in case of exposure during forced feeding period. Moreover, in France, a complete analysis of the contamination of food by FB1 revealed that avian kidney and liver may contain more than 100 μg FB1/kg of tissue (Leblanc, 2004), suggesting that human exposure to FB1 by the ingestion of food products derived from animals should probably be considered with more attention.

Synthesis During Meat Processing

As for other fusariotoxins, fumonisin production cannot occur on processed meats due to water activity required for toxinogenesis (Le Bars et al., 1994).

Other Toxins

Citrinin

Origin and Toxicity

Citrinin is produced by different *Aspergillus* (*A. terreus, A. carneus, A. niveus*) and *Penicillium* species (*P. citrinum, P. verrucosum, P. expansum*) (Sweeney & Dobson, 1998). It may also be produced by fungi belonging to *Monascus* genus (Blanc, Loret, & Goma, 1995). It has been found at levels ranging from few μg/kg to several mg/kg in barley, wheat, maize, but also in rice, nuts, dry fruits, apple juice, etc. (Abramson, Usleber, & Martlbauer, 1999; Abramson, Usleber, & Martlbauer, 2001; Vrabcheva, Usleber, Dietrich, & Martlbauer, 2000).

Citrinin is nephrotoxic in all animal species where it has been studied, leading to a time- and dose-dependant necrosis of renal tubules (Hanika, Carlton, & Tuite, 1983; Kogika, Hagikawa, & Mirandola, 1993; Manning, Brown, Wyatt, & Fletcher, 1985). This is mainly due to citrinin-mediated oxidative stress (Ribeiro, Chagas, Campello, & Klüppel, 1997).

Metabolism and Meat Contamination

Administration of labelled toxin demonstrated that citrinin is only weakly absorbed after oral administration and quickly eliminated in urine and faeces, at least in rodents (Phillips, Berndt, & Hayes, 1979). In poultry, the administration of a contaminated diet containing 440 ppm of citrinin did not allow the detection of residual contamination in muscles, whereas only weak amounts of the toxin were found in liver of exposed animals. Lower doses (110–330 ppm) did not lead to residual contamination of tissues (Kirby, Nelson, Halley, & Beasley, 1987). Therefore, due to the natural contamination levels observed in feeds (Abramson, Mills, Marquardt, & Frohlich, 1997), the risk of contamination of muscles seems very low.

Although citrinin-producing fungal strains have been isolated from dry cured meat products (El Kady et al., 1994; Wu, Ayres, & Koehler, 1974b) and it has been demonstrated that citrinin production may occur on dry cured meat (Bailly et al., 2005; Wu, Ayres, & Koehler, 1974a), no data are available on citrinin content in meat products, despite this toxin has been suspected to play a role in Balkan endemic nephropathy (Pfohl-Lezkowicz, Petkova-Bocharova, Chernozemsky, & Castegnaro, 2002) and is mutagenic (Sabater-Vilar, Maas, & Fink-Gremmels, 1999). However, stability studies demonstrated that this mycotoxin is only partially stable in cured ham, as already demonstrated in other animal-derived foods (Bailly, Querin, Bailly, Benard, & Guerre, 2002; Bailly et al., 2005). Nevertheless, it may be of interest to develop methods able to quantify a possible contamination of processed meat with citrinin.

Cyclopiazonic Acid

Origin and Toxicity

Cylopiazonic acid (CPA) was first isolated from culture of *Penicillium cyclopium* but has also been shown to be produced by several species of *Aspergillus* and *Penicillium* such as *A. flavus*, *A. tamarii* or *P. camemberti* (Le Bars, 1979; Martins & Martins, 1999). Therefore, CPA has been detected in many foods and especially cheeses (Le Bars, 1990), even though only few cases of intoxication have been described. However, retrospective analysis of "Turkey X disease" performed in 1986 by Cole suggested that clinical signs were not all typical of aflatoxicosis. He thus tried to demonstrate a possible role for cyclopiazonic acid in this affection. For instance, opisthotonos originally described in "Turkey X disease" can be reproduced by administration of a high dose of cyclopiazonic acid but not by ingestion of aflatoxin (Cole, 1986). Cyclopiazonic acid is a specific inhibitor of Ca2+ ATPase pump of endoplasmic reticulum (Seidler, Jona, Vegh, & Martonosi, 1989) that plays a key role in muscular contraction and relaxation (Nishie, Cole & Dorner, 1986). Principal target organs of cyclopiazonic acid in mammals are gastrointestinal tract, liver and kidney (Lomax, Cole, & Dorner, 1984; Nishie, Cole, & Dorner, 1985). Mains symptoms observed after acute intoxication with CPA are nervous signs with eyelid ptosis, ataxia with hypothermia, tremors and convulsions (Nishie et al., 1985).

Metabolism and Meat Contamination

Tissue transfer in muscle was characterized after oral administration of 0.5, 5 and 10 mg/kg b.w. using this HPLC quantification. The highest levels of contamination were found in muscle 3 h after administration. For birds fed 0.5 and 5 mg/kg b.w., the toxin was rapidly eliminated from meat in 24–48 h (Norred, Porter, Dorner, & Cole, 1988).

As for citrinin, no survey is available concerning CPA contamination of meat products. It has been demonstrated that CPA-producing strains could be isolated

from processed meats (Tabuc et al., 2004; Lopez-Diaz, Santos, Garcia-Lopez, & Otero, 2001; Sosa et al., 2002). Moreover, it has been showed that toxigenic strains of *Penicillium* were able to produce the toxin on meat products and that the toxin was stable on that substrate since more than 80% of the initial contamination was still recoverable after 8 days of incubation (Bailly et al., 2005). These results suggest that an accumulation of a relative high level of CPA could be observed on cured meat after contamination and development of toxigenic strains. Due to cyclopiazonic toxicity and its suspected role in "Kodua poisoning" in humans (Anthony, Janardhanan, & Shukla, 2003; Lalitha Rao & Husain, 1985), fungal strains used in meat processing should be tested for their ability to produce cyclopiazonic acid before use on commercial product. This recommendation is in agreement with previous one concerning the use of fungal starters in cheese (J. Le Bars & P. Le Bars, 1998). The development of micellar capillary electrophoresis for the detection of toxigenic mould strains may represent a useful alternative to classical analysis. It has already been applied to fungal strains isolated from cured meat and allowed multi-detection of mycotoxins such as CPA but also aflatoxin B1 (Martin, Jurado, Rodriguez, Nunez, & Cordoba, 2004). It appears also important to develop or adapt existing analytical methods to allow the final control of processed meats.

Conclusion

Mycotoxins are widely found contaminants of cereals and other vegetal products. When contaminated feeds are distributed to farm animals, mycotoxin may be found as residues in edible parts of the animals. For most important toxins, the available data on absorption, distribution within animal organism and metabolism revealed that mainly aflatoxins and ochratoxin A may be found at significant levels in muscles and muscle foods.

Mycotoxin contamination of meat may also result from toxigenic mould development during ripening and ageing. It may lead to production and accumulation of toxins such as citrinin or cyclopiazonic acid for which only few, if any, methods were set up for meat control. Even if the toxicity of such molecules appears less important than the previous ones, their possible implication in human diseases or syndrome should lead to the implementation of methods able to control contamination of processed meat.

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Chapter 5
Transmissible Spongiform Encephalopathy and Meat Safety

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Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) comprise a wide-ranging group of neurodegenerative diseases found in animals and humans. They have diverse causes and geographical distributions, but have similar pathological features, transmissibility and, are ultimately, fatal. Central to all TSEs is the presence of an abnormal form of a normal host protein, namely the prion protein. Because of their potential transmissibility, these diseases have wide public health ramifications.

In humans several forms of prion disease have been described, which can be divided into three groups related to causation such as idiopathic (sporadic CJD), acquired (iatrogenic CJD, variant CJD and kuru) and genetic forms of the disease (Table 5.1). Variant CJD (vCJD) has been shown to be caused by the same agent as bovine spongiform encephalopathy (BSE) in cattle and is most likely to have been transmitted to humans as a result of contamination of meat and meat products in the human diet between 1980 and 1996 (Bruce et al., 1997; Hill et al., 1997; Ferguson, Donnelly, Woolhouse, & Anderson, 1997; Scott et al., 1999). There are other prion diseases in animals that may form part of the human diet, for example, sheep and deer; however, there is no evidence to date that these animal prion diseases have transmitted to humans. Because of the transmissibility of these diseases and so their potential public health risk, surveillance (human and animal) and public health measures are necessary.

Scrapie in sheep has been described in Western Europe for over 200 years, although there is no evidence that it has spread to humans. Bovine spongiform encephalopathy (BSE) was first confirmed in the United Kingdom in 1986, though is thought to have been present, but undetected, in the 1970 s and early 1980 s. The development of the BSE epidemic in the United Kingdom resulted from the use of

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Table 5.1 Human prion diseases

Origin	Disease	Notes
IDIOPATHIC	Sporadic Creutzfeldt–Jakob disease (sCJD)	Worldwide distribution, disease of middle aged and elderly. Commonest form of human prion disease, but relatively rare, annual incidence 1–2 cases per million
ACQUIRED	Variant Creutzfeldt–Jakob disease (vCJD)	Found predominantly in the United Kingdom, disease of young. Caused by exposure to BSE
	Iatrogenic CJD	Due to transmission of *sporadic* CJD via neurosurgical instruments (4 cases), EEG depth electrodes (2), corneal transplant (2), human-derived growth hormone (194), gonadotrophin (4) treatment and human dura mater grafts (196*)
		Recently four cases** of *variant* CJD transmission via blood transfusion
	Kuru	Confined to Fore region of Papua New Guinea, spread via ritual endo-cannibalism, banned in late 1960 s. Few incident cases still occurring.
GENETIC	Specific types include	Due to autosomal dominant mutations of the prion protein gene (*PRNP*)
	Gerstmann Staussler Scheinker Syndrome (GSSS)	A genetic prion disease
	Fatal Familial Insomnia (FFI)	A very rare genetic prion disease

*123 cases in Japan, 13 in France, 10 in Spain and 7 in United Kingdom.
** Three resulted in clinical vCJD and 1 with evidence of infection (abnormal prion protein in spleen and lymph node) at time of death, but no clinical features of vCJD.

infectious meat and bone meal (MBM) in cattle feed. The additional use of infectious MBM in the feed of other animals resulted in TSEs in cats (feline spongiform encephalopathy) and in exotic ungulates and carnivores residing in zoos. The cause of BSE is uncertain, though it is speculated that it may have arisen as a spontaneous novel prion disease or as a result of a new mutation affecting the prion protein in cattle or in sheep (Horn, 2001; Inquiry Report, 2000). Chronic Wasting Disease (CWD) has been detected in wild and domestic deer and elk in the United States and appears to be spreading geographically and increasing in incidence (Williams, 2005). Table 5.2 summarizes animal prion diseases.

The Molecular Nature of Prion Diseases

The most characteristic and specific feature of prion diseases is the deposition of an abnormal form of a host protein, the prion protein, predominantly in the brain and central nervous system, but also elsewhere in body, depending on the

Table 5.2 Animal prion diseases

Disease	Notes
Scrapie	Disease of sheep and goats. Known in Western Europe for over 200 years
Bovine spongiform encephalopathy (BSE)	Primarily affected cattle. Transmission to feline species and exotic ungulates using the same feed as cattle
Feline spongiform encephalopathy (FSE)	Prion disease caused by BSE transmission to feline species via infected feed
Chronic wasting disease (CWD)	Disease of deer and elk species in North America
Transmissible mink encephalopathy (TME)	Disease of farmed mink
Atypical scrapie	Recently identified atypical form of scrapie in sheep
Bovine amyloid spongiform encephalopathy (BASE)	Recently described atypical form of bovine prion disease

disease type. The underlying pathological changes in the brain are neurodegenerative in nature, involving neuronal loss, spongiform change and astrocytosis (DeArmond, Ironside, Bouzamondo-Bernstein, Peretz, & Fraser, 2004). Prion protein (PrP^C) is a normal cellular protein encoded, in humans, by *PRNP*, the prion protein gene on chromosome 20. It is expressed predominantly in the brain, particularly in neurones and to a lesser extent elsewhere throughout the body, though its precise function is uncertain. Prion diseases are associated with a post-translational conformational change in PrP^C to an abnormal form of the protein, PrP^{Sc} (Prusiner, 2004a). This results in changes in the physicobiochemical properties of the molecule, which produces an increased resistance to degradation by biological and physical agents, including those used in the routine decontamination of instruments used for invasive medical procedures. The exact relationship of PrP^{Sc}, infectivity and the pathophysiology of clinical disease remains uncertain; the precise relationship between prion protein conversion or its tissue deposition and neuronal damage is not established. The prion hypothesis, which is the dominant view, proposes that PrP^{Sc} is either the agent of infectivity or the most significant component of it and the direct cause of disease, while other authorities suggest that there are other factors, even possibly that a small nucleic acid-based agent may be the causative agent (Diringer, 2001; Manuelidis, 2003). According to the prion hypothesis, sporadic, spontaneous, forms of the disease result from the normal form of PrP^C undergoing conformational change, either spontaneously or as a result of a *PRNP* somatic mutation, to the abnormal form (PrP^{Sc}) and so providing a template for further conversion of PrP^C to PrP^{Sc}. In the acquired forms of the disease, the PrP^{Sc} introduced into the host acts as a template for conversion of host PrP^C to PrP^{Sc} (Prusiner, 2004a; Aguzzi, Sigurdson, & Heikenwaelder, 2008). Genetic forms of the disease result from pathogenic mutations of *PRNP* (Kovacs et al., 2002).

Human prion diseases can be classified on a protein basis according to the PrP^{Sc} type (dependent on the size of the protease resistant fragment after degradation with proteases) and the ratio of glycoforms found; the most commonly used protein classification being into Type I, Type IIA and Type IIB (Parchi et al., 1996; Head et al., 2004). The exact significance of this protein classification is unclear and it is not entirely straightforward as different protein types can be found in one brain (Head et al., 2004). Polymorphisms in *PRNP* play an important role in susceptibility to developing disease, the incubation period (in acquired forms) and may influence the resulting clinico-pathological phenotype (Parchi et al., 1999). At codon 129 of *PRNP*, an individual may code either for methionine (M) or for valine (V); therefore each person is either heterozygous (MV) or homozygous (MM, VV). There is some geographic variation in the distribution of these different genotypes in normal populations (Nurmi et al., 2003). In the acquired prion diseases the human growth CJD recipients and kuru, MM individuals had shorter incubation period and increased susceptibility to disease compared with individuals with MV and VV genotypes (Cervenakova et al., 1998; Clarke & Ghani, 2005; Brandel et al., 2003; Huillard d'Aignaux et al., 2002). In addition in vCJD, all the UK cases tested to date (88%) have been found to be methionine homozygotes. A clinicopathological and molecular classification of sporadic CJD, based on PrP type, *PRNP*-129 genotype, clinical and pathological features was proposed by Parchi and colleagues and is in current use (Parchi et al., 1999).

Prion diseases are transmissible, including those with genetic causes, which give rise to a number of important public health concerns. In general, transmissibility is associated with PrP^{Sc} and the generally accepted view is that PrP^{Sc} is either the infectious agent or its major component (Prusiner, 2004b). However, the picture remains unclear as non-infectious forms of PrP^{Sc} have been shown in experimental models while in others, in the presence of infection or disease, no or little PrP^{Sc} has been detected (Barron et al., 2007). There is no simple diagnostic or screening test for human prion diseases, for example a blood test. Methods of detecting infectivity in tissues either involve laboratory animal transmission experiments, which provide definitive evidence of transmissibility, but are slow and expensive, or identify PrP^{Sc} in tissues, which are quicker and cheaper, but indirect (using PrP^{Sc} as a marker of infectivity). Both require appropriate tissue samples, which, in the case of human brain, are not easy to obtain in life and may be difficult to obtain after death, because of issues surrounding post-mortems and consent.

In this chapter we attempt to provide a description of variant CJD and how it relates to other human prion diseases, the relationship of bovine spongiform encephalopathy (BSE) to variant CJD, the public health measures put in place to prevent transmission and the human public health implications of other animal TSEs.

Epidemiology of Variant CJD and Other Human TSEs

Variant CJD

The UK National CJD Surveillance Unit (NCJDSU), Edinburgh, was established by the Department of Health in 1990 following the Southwood Committee Report (www.cjd.ed.ac.uk). At its inception, the principal aim of the NCJDSU was to identify any changes in CJD in the United Kingdom, in the wake of the cattle BSE epidemic, that could indicate transmission of BSE to man. In 1996, the NCJDSU reported the emergence of a previously unrecognised form of CJD, known as variant CJD (vCJD) (Will et al., 1996). The NCJDSU continues with UK CJD surveillance, identifying all cases of human prion disease, along with associated research: particularly to examine risk factors for all UK cases of sporadic and variant CJD, to investigate the geographic distribution of CJD, to identify mechanisms of transmission of BSE to humans, to establish short and long term trends, to evaluate potential risks of onward transmission, to identify novel forms of human prion diseases and to evaluate case definitions and diagnostic tests.

Up to 31 December 2007, 166 cases of definite or probable vCJD had been identified in the United Kingdom. Figure 5.1 shows the number of cases of vCJD in the United Kingdom by year. Seventy-three (44%) of the 166 cases were women. The median age at onset of disease was 26 years and the median age at death 28 years (compared with 66 years for the median age at onset and 67 years for the median age at death for sporadic CJD). The youngest case was aged 12 years at onset, while the oldest case was aged 74 years. To date, no case of vCJD has been identified in the United Kingdom in individuals born after 1989. The age- and sex-specific mortality rates for vCJD over the time period 1 May 1995 to 31 December 2006 are shown in Fig. 5.2. The median duration of

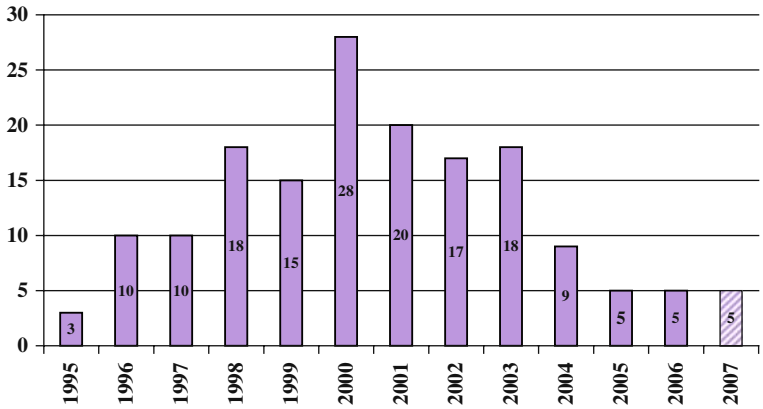

Fig. 5.1 The number of deaths per year of variant CJD in the United Kingdom

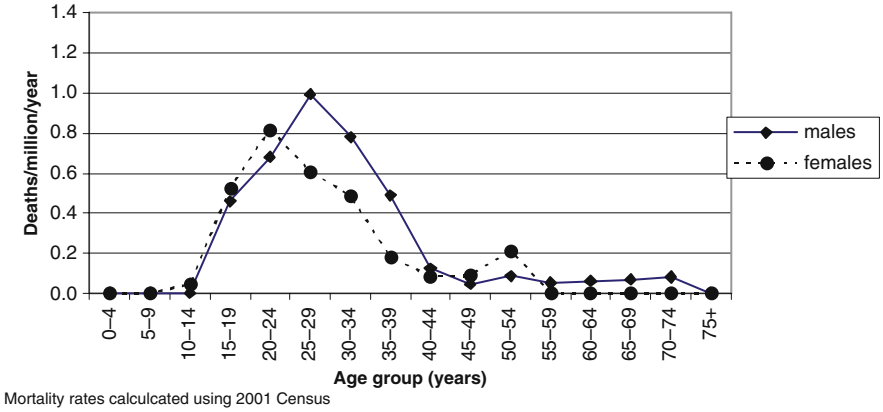

Fig. 5.2 Age- and sex-specific mortality rates of vCJD in the United Kingdom from 1 May 1995 to 31 December 2006

illness from the onset of first symptoms to death was 14 months (range 6–40) compared with the median duration of illness for cases of sporadic CJD, which was 4 months (range 1–74).

In addition to the 166 cases of vCJD in the United Kingdom, there have been 41 vCJD cases diagnosed elsewhere in the world (worldwide total = 207), a small proportion of which (6) have resided in the United Kingdom for greater than 6 months between 1980 and 1996, which is considered the at risk period in the United Kingdom for exposure to BSE in diet. After the United Kingdom, France has reported the greatest number of cases, 23, but modelling predicts a limited size to the epidemic, and with a lower total than for the United Kingdom (Alperovitch & Will, 2002).

There have been four cases of transfusion association vCJD infection to date, who received blood from people who went on to develop symptoms of vCJD. Three of these individuals developed clinical vCJD (one diagnosed in 2003 and two in 2006), while the fourth died from causes unrelated to vCJD, but was found on post-mortem examination to have abnormal prion protein present in the spleen and a lymph node (2004) (Llewelyn et al., 2004; Peden, Head, Ritchie, Bell, & Ironside, 2004; Wroe et al., 2006; HPA, 2007). There is no evidence of transmission of vCJD through plasma products, surgery or dental transmission, although a risk of transmission remains with each of these routes (Ward et al., 2006).

Results from modelling the underlying incidence of diagnoses and deaths indicate that the primary epidemic reached a peak in the year 2000 (NCJDSU Annual Report, www.cjd.ed.ac.uk). It is important to note that although a peak has been passed, it is possible that there will be future peaks, possibly in other genetic groups. There is also the possibility of ongoing person-to-person spread as seen with four cases of transfusion association vCJD infection to date, who received blood from earlier cases.

To date all the vCJD cases tested have been methionine homozygous at codon 129 of the prion protein gene. Follow-up studies on patients who developed Kuru or iatrogenic CJD (human-derived growth hormone recipients) suggest that individuals who were homozygous for methionine at codon 129 developed clinical disease sooner than heterozygote (MV) or valine homozygote (VV) individuals, who have appeared less susceptible with longer incubation periods, greater than 40 years in some cases (Cervenakova et al., 1998; Clarke & Ghani, 2005; Brandel et al., 2003; Huillard d'Aignaux et al., 2002). There is no reason to believe that vCJD would behave differently from other human prion disease, therefore, it is likely that those with non-methionine homozygous codon 129 genotypes will develop clinical disease and/or sub-clinical infection. However, the numbers of cases are unlikely to be greater than those seen for methionine homozygous individuals. Evidence to support this comes from three sources. First, while all clinical cases of variant CJD tested have been methionine homozygous, the second reported instance of transfusion-transmitted variant CJD infection (who did not develop clinical vCJD, but died of other causes) was shown to have heterozygous (MV) at codon 129. In addition, two of the three patients who tested positive for abnormal protein in a retrospective study of appendices have been shown to be valine homozygous (Ironside et al., 2006). Lastly, experimental data in mouse models also suggests that heterozygosity (MV) or valine homozygosity at codon 129 predisposes to a prolonged period of sub-clinical disease (Bishop et al., 2006).

There have been many studies published with the aim of estimating the number of clinical and sub-clinical cases of vCJD using mathematical modelling techniques. At the start of the vCJD epidemic, when uncertainties were greatest, the size of the epidemic was estimated to be in the millions (Ghani, Ferguson, Donnelly, Hagenaars, & Anderson, 1998). As the primary epidemic has progressed, the estimated number of clinical cases has reduced to less than 400 cases (Clarke & Ghani, 2005). However, from a public health viewpoint, it is not only the number of clinical cases that are of interest but also the number potentially infectious with sub-clinical or pre-clinical infection.

A key factor in determining the likelihood of onward transmission from person to person is estimating the prevalence of vCJD infection in the population. Without a simple diagnostic test, such as a blood test, this is difficult, complex and costly to undertake. A retrospective, tissue-based study using stored appendix and tonsil samples was performed to estimate the prevalence of vCJD in the United Kingdom. Three appendix samples out of a total of 12,674 samples tested were found to be positive for abnormal prion protein (Hilton et al., 2002, 2004). From these results mathematical modelling has predicted an estimate of between 3,000 and 5,000 infected people (95% CI: 520–13,440) in the United Kingdom, mainly in the 10–30-year age group. It is estimated that the majority of these will have sub-clinical infection (93–96%, 95% CI: 70–99%) with only 4–6% developing clinical disease (Clarke & Ghani, 2005). However, those with infection may (or may not) have the ability to

transmit the disease through invasive medical procedures, such as surgery, dentistry and through blood transfusion.

A further study is underway by the UK Health Protection Agency, the National Anonymous Tonsil Archive study, which is gathering tonsils removed through routine surgery in the United Kingdom for testing for abnormal prion protein (3000 tonsil pairs are included from the National Prion Unit, London). Interim results of 0 confirmed positives out of 45,000 tonsils tested have not altered estimates in the prevalence of vCJD in the UK population, mainly because the relatively large numbers of those tested were in birth cohorts born after the peak of the BSE outbreak, that is they were not considered to be at high risk of exposure to BSE (www.seac.gov.uk/summaries/seac100_summary.pdf).

Risk Factors for Variant CJD

Early in the vCJD epidemic, three factors predisposing to the disease became evident: residence in the United Kingdom, methionine homozygosity at codon 129 of the prion protein gene and the relative young age of the individuals compared with sporadic CJD. In addition to these, a range of hypotheses were generated concerning risk factors relating to possible routes of exposure to the BSE agent, to predisposing factors or to other unrelated possible causes of the disease, such as exposure to organophosphates. Possible routes of exposure to the BSE agent included the most likely, which was through diet, and others such as surgery, medicines, including vaccines, certain occupations having contact with cattle, meat or products manufactured from cattle/meat (for example farmers, abattoir workers, butchers and laboratory workers) and contact with animals. Factors that were considered as predisposing factors included social class, ethnicity and urban/rural residence.

A case–control study was carried out comparing the frequency of certain risk factors (including dietary, medical, surgical, occupational) in vCJD cases with the frequency in people without CJD ("controls"). The methodology of this study has been described in detail previously (Ward et al., 2006). The results showed that reported frequent consumption of beef and beef products thought likely to contain mechanically recovered or head meat, or both, including burgers and meat pies, was associated with increased risk of vCJD. There was no evidence of socio-economic differences between cases and controls to explain these findings. However, reported consumption of chicken was also more frequent in cases than controls, which may be explained by recall bias (relatives of cases remembering more detail than relatives of controls), that it was a chance finding, or there was circumstantial evidence that some chicken and pork products contained beef mechanically recovered meat. The study found no evidence of a high proportion of cases in the study being infected through occupational exposure, including farming, veterinary medicine, meat and catering industries and medical and laboratory workers, or through reported animal, pesticide or fertiliser exposure since 1980. The reported exposure of cases and controls to medical, surgical and related risk factors was similar, except for a small group of minor operations for

which cases reported more frequent exposure. This possibly due to under-reporting by relatives of controls. The findings of this study provide evidence supporting the dietary hypothesis as the main route of transmission of BSE to humans in the primary VCJD epidemic. However, they do not exclude the possibility of further "waves" of the epidemic occurring due to other risks, such as, through blood transfusion and surgery.

Cooper and Bird (2002a, 2002b, 2002c) attempted to quantify the UK dietary exposure to BSE using national surveys on diet and nutrition. They based their work on the assumption that the most likely exposure to dietary BSE was through beef mechanically recovered meat (MRM) and head meat used in burgers, sausages and other meat products. Approximately 90% of MRM and 80% of head meat was reportedly used in the production of burgers. The time period between 1980 and 1996 is considered the greatest "at risk" period for exposure to BSE in the diet in the United Kingdom. The highest consumption overall was in males in the 1940–1969 birth cohort, followed by the post-1969 and the pre-1940 cohorts. Although exposure through the dietary route explains to some extent the relatively young age of vCJD cases, an increased susceptibility and/or shorter incubation period is also needed to account fully for the age distribution.

The geographical distribution of cases of vCJD has remained of interest in terms of determining possible common exposures and so helping to elucidate possible risk factors. It was observed that individuals living in 1991 in the "North" of the country (Scotland, North, Yorkshire and Humberside, North West) were about one and a half times more likely to have developed vCJD than individuals who were living in the "South" (Wales, West Midlands, East Midlands, East Anglia, South West, South East). The rate ratio controlling for age and sex is 1.55 (95% CI, 1.14, 2.12). The difference remains when the analysis is adjusted for socio-economic status, urban/rural mix and population density. Although regional variations in diet might explain these observed differences, results of dietary analyses were inconsistent (Cousens, 2001).

The Leicestershire cluster of five cases remains the only statistically significant cluster of cases to date. The results of the investigation of the cause of the Leicestershire cluster revealed that local butchers split bovine heads and removed the brains, which was an old-fashioned, but legal, practice at the time. The same knives and other instruments that were used to split the head and remove the brains were also used to obtain cuts of meat from the rest of the carcass, which may have resulted in cross-contamination of the cuts of meat with the BSE agent, which has highest titres in the brain and central nervous system (www.hpa.org.uk/cdr/archives/2001/cdr1201.pdf). Investigations in other areas did not reveal any suggestion of similar practices as a cause of cases.

If we assume that the dietary transmission BSE was virtually removed in the United Kingdom by measures put in place by 1996, the remaining question is whether there will be further cases as a result of secondary transmission. The tissue distribution of vCJD beyond neural tissue (mainly lymphoreticular tissues) means that the secondary transmission of vCJD through invasive medical procedures, transfusion of blood components and plasma products, dentistry

and organ and tissue transplantation remains a possibility and a concern. Mathematical models to predict the chances of self-sustaining epidemics from secondary transmission have been undertaken. Some scenarios involving blood transfusion have revealed that self-sustaining epidemics were possible, but, biologically implausible and, therefore, unlikely. Public health interventions, such as leucodepletion, were effective (Clarke, Will, & Ghani, 2007). Other models also predicted that vCJD would not become endemic by blood transfusion alone (Dietz et al., 2007). Scenarios involving surgery showed that self-sustaining epidemics were possible and that key factors determining the scale of the epidemic were the number of times an instrument was re-used, the infectivity and the effectiveness of cleaning of the instruments (Garske, Ward, Clarke, Will, & Ghani, 2006).

Clinical Features of vCJD and Other Human TSEs

Human TSEs are characterised clinically by a progressive and uniformly fatal encephalopathy. Even when the disease is acquired by infection, there are no typical infection indicators: there is no pyrexia, the peripheral blood white cell count, ESR and CRP remain normal and there is no detectable antibody response. Indeed, the illness is entirely neurological; even when other tissues may be pathologically affected (for example lymphoreticular involvement in vCJD), there is no clinical evidence of dysfunction outside the CNS. The clinical features vary from case to case, depending on the type of disease, the genetic characteristics of the affected person and other factors. Cognitive impairment progressing to a global dementia, cerebellar ataxia and involuntary movements (particularly myoclonus) are common features. Most cases present as a rapidly progressive encephalopathy dominated by dementia and this is certainly so for typical sporadic CJD. However, there are other presentations including a progressive cerebellar ataxia (seen in some cases of sCJD, CJD related to human growth hormone and in some genetic prion disease).

Variant CJD, however, typically presents with psychiatric or behavioural disturbances; specifically neurological features tend to develop after several months. The initial clinical picture is typically non-specific and suggestive of depression. The clinical features in the first hundred UK cases have been analysed in detail: differentiation from much more common, less serious illnesses is highly problematic and early clinical diagnosis may be impossible (Spencer, Knight, & Will, 2002).

The first definitive neurological features are often those of cerebellar ataxia. Sensory symptoms, often painful, and involuntary movements such as chorea or dystonia are relatively common (Macleod, Stewart, Zeidler, Will, & Knight, 2002). As the disease progresses, severe cognitive impairment develops and the patient becomes increasingly dependent on others for all activities. Terminally, the patient is bedbound and severely demented, often with myoclonus. The illness takes a rapidly progressive course with a median duration, from first

symptom to death, of only around 14 months. While sCJD typically affects the middle aged and elderly, vCJD has mostly affected the relatively young (the median age at onset in the United Kingdom being 26 years), as discussed above. Most UK cases, and all cases elsewhere, are thought to have resulted from dietary contamination with BSE, but three UK cases have resulted from blood transfusion. The clinical features of vCJD have not varied with age of onset or with the mode of infection.

Diagnosis of vCJD and Other Human TSEs

Diagnosis of all human TSEs can be viewed in three parts: suspecting the diagnosis, excluding other possibilities and utilising supportive diagnostic tests. Suspecting the diagnosis depends on familiarity with the typical clinical features. The diagnosis of genetic human prion disease should be suggested by a relevant (autosomal dominant) family history, however, this may be absent in around 40% of cases (Kovacs et al., 2002, 2005). Analysis for relevant *PRNP* mutations can be performed on any suitable tissue, including blood, by a laboratory with relevant expertise and following appropriate pre-test counselling.

Iatrogenic CJD should be suspected on the basis of a previous relevant exposure, for example to cadaveric-derived hGH treatment, a dura mater graft or – in the cases of vCJD – a blood transfusion derived from an identified vCJD donor. In the early stages of the illness a TSE may not be suspected as other, much commoner, possibilities suggest themselves. This is particularly so in vCJD, with its tendency to affect the relatively young and its generally non-specific presentation. Many alternative diagnoses may require exclusion and a full discussion is beyond scope of this discussion. Currently, there are no non-invasive absolute clinical diagnostic tests for human TSEs. Definite diagnosis requires neuropathological examination of brain tissue; cerebral biopsy is rarely indicated and this therefore usually takes place at autopsy. However, there are several useful supportive tests: cerebral MRI, EEG, CSF protein analysis and tonsil biopsy.

The cerebral MRI has two important roles. First, it is necessary to exclude other possible diagnoses for an encephalopathic illness. Second, there are MRI features which may suggest, or support, a diagnosis of human prion disease. In sCJD, high signal may be seen in the anterior basal ganglia in about two thirds of cases, and sometimes also in areas of the cerebral cortex (Collie et al., 2001). In vCJD, high signal may be seen in the posterior thalamus, the so-called "pulvinar sign", in around 90% of cases (Collie et al., 2003). These abnormalities are not unique to prion diseases, but other causes are usually identifiable from the clinical context. FLAIR and DWI are the most sensitive MRI sequences for these changes in prion disease. A protocol for MRI investigation of prion disease has been published (Collie et al., 2001).

The cerebrospinal fluid (CSF) is likely to be examined in most cases, in the process of considering other possible diagnoses (for example, related to malignancy,

inflammation or viral, fungal and bacterial infections). There is no CSF pleocytosis in prion disease and while the total protein level is often elevated, this is a very non-specific finding (Green et al., 2001). However, certain specific proteins are potentially helpful in diagnosis: 14-3-3, S100b and tau. It is important to understand that these are all normal CNS proteins that may be found in the CSF in a wide variety of circumstances, including trauma, stroke and other infective or neurodegenerative conditions. There sensitivity for prion diseases depends on the destructive and reactive brain processes characteristic of prion disease; the sensitivity varies according to the type of prion disease. Their specificity for prion diseases depends on their clinical context, including the prior exclusion of other potentially relevant illnesses. 14-3-3 is a relatively sensitive test for sCJD, being found in around 90% of cases (Zerr et al., 2000; Green et al., 2001); when using the WHO diagnostic criteria a positive 14-3-3 test elevates a possible case of sCJD to a probable one. The CSF 14-3-3 test has a significantly lower sensitivity for vCJD compared with sCJD and, consequently, it has a relatively low negative predictive value; CSF tau is a relatively more sensitive test (Green et al., 2001).

There are characteristic EEG changes (generalised periodic discharges) associated with sCJD which support the diagnosis and are included in the WHO diagnostic criteria. However, these are not usually seen in vCJD; in two cases such characteristic periodic discharges have been reported, but only in the very late disease stage (Yamada, 2006; Binelli et al., 2006). As there is significant deposition of the abnormal prion protein (PrP^{Sc}) in lymphoreticular tissues in vCJD (unlike in sCJD, gCJD and non-variant iCJD), tonsil biopsy is a useful investigation in suspect vCJD. The tonsil is relatively accessible and a positive finding reflects to a disease-specific abnormality (unlike the other supporting tests). However, neuropathological examination is still necessary for an absolutely definite diagnosis. While it is arguably a relatively minor undertaking, tonsil biopsy has a potential morbidity and it is a clinical decision as to whether it should be done in a given case. The absence of the MRI pulvinar sign or an unusual clinical picture is obvious situations where it could be useful. However, in the United Kingdom, most clinically typical cases with the MRI pulvinar sign have not undergone tonsil biopsy. No UK case that has fulfilled the present WHO criteria for probable vCJD, who has then undergone autopsy, has turned out to have another diagnosis (NCJDSU data). In countries considering their first reported case, the additional diagnostic support of a tonsil biopsy has often been carefully considered partly as the clinicians had no previous experience of diagnosing vCJD and partly because of the public health and other significance of detecting this illness in their population.

BSE Epidemic and Relationship to Variant CJD

BSE occurs in adult animals in both sexes, typically in animals aged 5 years and more. It is a neurological disease in which affected animals show signs that include changes in mental state, abnormalities of posture and movement and of

sensation. The clinical disease usually lasts for several weeks and it is invariably progressive and fatal. The average incubation period of BSE is 5 years, and only very rarely do animals under 3 years of age display symptoms.

BSE was first confirmed in the United Kingdom in 1986, though it is thought to have been present, but undetected, in the 1970 s and early 1980 s. The epidemic of clinical cases in the United Kingdom reached a peak in 1992 with over 37,000 cases identified and a total so far of over 180,000 cases. With measures put in place (see below), the epidemic has been in decline for many years with 114 cases in 2006, (http://www.oie.int/eng/info/en_esbru.htm). These represent the number of clinical cases; however, there have been estimations that many more infected cattle may have entered the food chain as a result of slaughter of pre-clinical cases of BSE (Anderson et al., 1996).

Since 1989 cases of BSE have also been confirmed in many European Union countries (Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, the Netherlands, Poland, Portugal. Slovakia, Slovenia, Spain, Sweden) and, also, in non EU countries (Canada, Israel Japan, Liechtenstein, Switzerland and the United States). However, the scale of the BSE epidemics in each of these countries has not reached that of the United Kingdom, with the highest numbers of cases in the Republic of Ireland, Portugal and France being between 980 and 1,600 cases of BSE. In addition, the number of cases of BSE in cattle identified depends on methods of identification, which were introduced at different times, and in different ways, in different countries. The number of BSE cattle reported also depends on whether surveillance is passive or active and the population tested: clinically suspect cases, ill animals, animals discovered dead or healthy animals slaughtered for human consumption. In 2001, new testing rules were introduced in the EU, including the need for post-mortem testing of all apparently healthy cattle over 30 months of age at slaughter for human consumption. The true figures for BSE in many countries in the past remain subject to doubt; it is clear that numbers rose significantly in a number of EU countries after the introduction of the more comprehensive testing policy in 2001. The figures for BSE in different countries can be obtained through OIE (The World Organisation for Animal Health) (www.oie.int).

In 1996, a new form of human prion disease was first recognised in the United Kingdom, initially termed "new variant CJD" and now known as variant CJD or vCJD (Will et al., 1996). It was identified by its distinct clinical, neuropathological and epidemiological features as compared with other forms of CJD, particularly sporadic CJD (sCJD). While sporadic CJD typically presents with cognitive impairment and has a median age of presentation of 67 years, vCJD typically has a psychiatric presentation and is found in the 20- to 30-year age group. The geographical temporal association between BSE and vCJD led to the hypothesis that vCJD was caused by transmission of BSE from cattle to humans. Transmission studies in mice provided the proof that vCJD was caused by the same "agent" as BSE and was distinct from other human prion diseases (Bruce et al., 1997; Hill et al., 1997; Ferguson et al., 1997; Scott

et al., 1999). Since 1996, other countries have also had cases of vCJD (see above). The risk to a particular country of vCJD not only depends on the incidence of BSE within the country, but perhaps more importantly on imports of bovine material, BSE-affected cattle and infected cattle feed from high-risk countries (particularly the UK) (Alperovitch & Will, 2002).

Although the most probable route of transmission of BSE to humans was through diet, other possibilities were considered, such as through medicines, including vaccines, that contained products prepared from cattle and through contact with cattle (butchers, farmers, abattoir workers). In addition, transmission through an intermediate species, such as cats, and transmission to cattle and humans from a third species, such as from scrapie in sheep, were also considered as possible methods of transmission. A UK case–control study was established in 1998 to examine these and other risk factors for vCJD (see above, Ward et al., 2006).

Public Health Measures Implemented to Reduce Transmission of BSE

During the late 1980 s, as the BSE epidemic progressed, there was considerable public concern about BSE and the safety of British meat. The UK Government set up an independent committee (SEAC) of leading experts to ensure that it received the best possible scientific advice surrounding TSEs. Control measures were introduced and strengthened in the United Kingdom in order to reduce the risk of people eating beef and meat products that might be infected with BSE. The aim of these measures was to reduce the risk to a level that was acceptable on balance of factors such as practicality and cost, while at the same time recognising that the risk from BSE could not be removed completely. In January 1998, the UK "BSE Inquiry" was set up to "establish and review the history of the emergence and identification of BSE and new variant CJD in the United Kingdom, and of the action taken in response to it up to 20 March 1996; to reach conclusions on the adequacy of that response, taking into account the state of knowledge at the time" (www.bseinquiry.gov.uk). The UK Food Standards Agency was established in 2000 in order to protect public health in relation to food. One of its key aims is to ensure that controls in relation to TSEs minimise the potential risk from eating beef and sheep meat and are based on the latest scientific knowledge (www.food.gov.uk).

The controls that were put in place can be divided into those that were primarily aimed at reducing the spread of BSE between animals and so preventing new, incident cases of BSE (meat and bone meal feed ban) and those that were aimed at reducing the transmission of BSE from cattle to humans (BSE detection in animals, the removal of specified risk material and certain mechanically recovered meat from the human food chain). They are summarised below.

Prevention of Spread of BSE Between Animals

The UK government BSE Inquiry concluded that the development of the BSE epidemic resulted from the use of infectious meat and bone meal (MBM) in cattle feed. The MBM had been produced by the rendering (industrial cooking) of carcases of cattle infected with BSE (www.bseinquiry.gov.uk).

In 1988, a ban on the feeding of meat and bone meal (MBM) to ruminants was introduced in the United Kingdom. While this and other controls (see below) markedly reduced the risk of infection in the United Kingdom, cattle feed contaminated with pig or poultry feed containing MBM continued to infect cattle after the 1988 ban. However, in August 1996 this was extended to cover the feeding of MBM to all farm animals and an EU – wide ban was introduced in 2001. The regulations prohibit the use of mammalian protein in feed to ruminant animals, and the incorporation of mammalian MBM in any farm livestock feed.

Detection of BSE in Cattle

Since 1988, under the UK Compulsory Slaughter and Compensation Scheme, all cattle suspected of suffering from BSE have been slaughtered and sent for diagnosis. All cattle with suspect BSE are destroyed by incineration. In addition, all adult cattle presented for slaughter are inspected by veterinary surgeons to make sure that no suspect cases of BSE enter the human food chain. Animal identification tracing of movement also plays a role in the measures against BSE.

Between 1996 and November 2005 in the United Kingdom, there was a ban on any cattle aged over 30 months from entering the food chain (the "Over Thirty Months Rule"). Cattle younger than 30 months were considered unlikely to carry a significant amount of BSE infectivity. From November 2005, following a review by the UK Food Standards Agency, a system of BSE testing was introduced for slaughtered cattle aged over 30 months, intended for human consumption. This replaced the previous outright ban and only cattle testing negative for BSE can enter the human food chain. The additional risk to the public resulting from this change in practice was considered to be extremely low.

To ensure that controls are enforced in the United Kingdom, the State Veterinary Service carries out inspection of farms, while the Meat Hygiene Service inspects abattoirs and cutting plants.

The Removal of High-Risk Material from the Food Chain

Tissues known as Specified Risk Material (SRM), which include the tonsils, intestine, brain and spinal cord, are considered most likely to carry BSE infectivity. Therefore, since 1989 controls (by law) have existed in the United

Kingdom to prevent these tissues of cattle, sheep and goats from entering the human food chain. If an animal with pre-clinical BSE, that is with no clinical signs, entered the human food chain, these controls are estimated to remove approximately 99% of potential infectivity. In 2000 harmonised SRM controls were introduced across all EU Member States.

The controls have been regularly reviewed and strengthened in line with developing scientific evidence. For example, in December 1997 bone-in-beef and beef bones were excluded from the human food chain to protect public health from the perceived risk of BSE infectivity. Because of the decline in the BSE, the ban was reviewed and lifted towards the end of 1999, though it was retained for manufacturing uses of both bone-in-beef and beef bones. Cattle born before August 1996, when a reinforced ban was introduced on animal feed containing meat and bone meal, are permanently excluded from the food chain in the United Kingdom.

Prevention of Transmission of BSE from Cattle to Humans

Mechanically recovered meat (MRM or mechanically separated meat, MSM) was derived from the flesh-bearing bones and carcass remnants of cattle, sheep, pigs and poultry. During the early 1960 s automated high-pressure devices were developed whereby residual material, which was difficult to remove by hand, could be removed from bones and carcasses in a puree form. The vertebral column was often used for the production of MRM and this resulted in the risk of cross contamination of MRM by spinal cord and dorsal root ganglia which potentially contained high titres of BSE infectivity. MRM was used in the preparation of various meat products, including "economy" burgers, sausages, meat pies, soups, some baby foods and prepared meals (www.bseinquiry.gov.uk). In 1995 the United Kingdom banned the use of cattle vertebral column in MRM. This ban was extended in 1998 in the United Kingdom to cover the vertebral column of all grazing animals. The production of MRM from all ruminant bones was subsequently prohibited throughout the EU.

Different countries introduced human dietary protection measures at different times. For example, animal CNS material was reportedly still being used in the preparation of certain sausages in Germany in 2000 (Lucker, Eigenbrodt, Wenisch, Leiser, & Bulte, 2000). Therefore, to minimise the risk of BSE in meat for human consumption in all EU countries, since 2001 EU-wide regulations laying down the rules for the prevention, control and eradication of certain TSEs have been in place. These were enforced by domestic legislation. In 2005, the "TSE Roadmap" (TSE Roadmap, COM (2005) 322 FINAL) was produced by the European Commission. This provided an outline of possible future changes to EU measures on BSE in the short, medium and long term. Since 1995 the Commission has generated 70 primary and implementing acts setting out stringent measures to protect animal and human health at the Community

level. With indications of a favourable trend in the BSE epidemic, the goal for the coming years is to ensure relaxation of measures while assuring high level of food safety is maintained. Relaxation of measures is risk based and aims to reflect advances in technology and evolving scientific knowledge.

Treatment of Human Prion Diseases

Currently, there are no treatments proven to modify the disease course in human prion diseases: they are universally progressive and fatal. While it is important to remember that the precise pathogenesis of prion diseases remains unclear, there has been an increasing understanding of prion diseases, and this has suggested various possible treatment approaches (Head, Farquhar, Mabbott, & Fraser, 2001, Weissmann & Aguzzi, 2005).

There are many reports of experimental treatments based on in vitro and animal in vivo models; the animal data have been reported in a recent systematic review (Trevitt & Collinge, 2006). There are obvious potential problems in extrapolating from in vitro and in vivo models to human illness. Two particular problems relate to disease strain and disease stage. Animal models have often studied disease strains (like scrapie) that do not necessarily have relevance to human illnesses (such as vCJD, sCJD and gCJD). Many of the animal experiments have studied attack rate and incubation period following simultaneous, or near-simultaneous, administration of infection and treatment; this situation is quite different from that of treating clinically ill humans. Treatments in human prion disease have been reported, often in the form of brief case reports. The sum of such reports has been described in a systematic review published in 2008 (Stewart, Rydzewska, Keogh, & Knight, 2008). To date, there is only one report of a blinded, placebo-controlled trial of human disease treatment (Otto et al., 2004). Individual countries have embarked upon treatment trials, for example with aquamarine in the United States and the United Kingdom (www.clinicaltrials.gov: NCT00183092, www.ctu.mrc.ac.uk/studies/cjd.asp). A recent report describes the use of intraventricular PPS (Pentosan Polysulphate) in the United Kingdom (Bone, Belton, Walker, & Darbyshire, 2008).

There are a number of problems with the assessment of potential treatments in organised human trials. First, these diseases are rare International collaboration is probably necessary as mentioned in a recent editorial; such collaboration has begun with the EU Theraprion project (Pocchiari, Ladogana, Graziano, & Puopolo, 2008). Second, the diagnosis is presently usually made at a relatively advanced disease stage, with corresponding severe pathological abnormality and significant disability, raising therapeutic and ethical problems. The development of an appropriate, non-invasive, early diagnostic test would be an important aid for treatment and its assessment. There are also problems with the actual assessment of any treatment effect; complete cure would be obvious, but an unlikely result of initial treatments. The problem of assessment of

progression in serious brain disease is a familiar one to neurologists and there are no characterised paraclinical indices of disease progression. Survival time is an obvious measure. However, the precise identification of the onset of illness in some prion diseases can be difficult. In addition, there are a variety of factors that may influence the disease's natural history: disease strain, age at onset, sex and individual genotype (Pocchiari et al., 2004). These variations need to be allowed for by the prior division of treated patients into subgroups and this exacerbates the problem of disease rarity.

Other Animal TSEs and the Risk to Humans

Chronic wasting disease (CWD) is a prion disease of captive and wild deer and elk, first recognised in the United States in the 1960 s. Although CWD can appear to spread horizontally with environmental contamination playing an important role, its exact modes of transmission have not been clearly defined. Abnormal prion protein is found in lymphoid tissues and faecal or salivary shedding of the agent is plausible. Since the 1960 s, CWD has spread geographically in North America and increased in numbers. However, there is no evidence to date that CWD has been transmitted to humans, though surveillance continues, especially in the deer hunting communities (Prusiner, 2004c).

BSE has been demonstrated in a goat in France, although this was positive before the ban on feeding MBM to all farm animals came into force. The European Food Safety Authority considered the risk of exposure to BSE through consumption of goat meat by humans to be small (Eurosurveillance, 2005). Although BSE can be transmitted to sheep under experimental conditions, it has not been demonstrated in sheep (farmed or wild) to date. In addition, unusual or "atypical" forms of scrapie and BSE (BASE or bovine amyloidotic spongiform encephalopathy) have been demonstrated recently with the use of more sensitive biochemical assays in sheep and cattle, respectively (Yamakawa et al., 2003; Casalone et al., 2004; Benestad et al., 2003; Buschmann et al., 2004; Orge et al., 2004; Onnasch, Gunn, Bradshaw, Benestad, & Bassett, 2004; Gavier-Widén et al., 2004; De Bosschere, Roels, Benestad, & Vanopdenbosch, 2004). The extent of these forms of the diseases and how long they have been in existence remains unknown. Further work is continuing in this area.

Discussion

The BSE epidemic in the United Kingdom in the late 1980 s and early 1990 s had a devastating effect on the farming, meat and food industries in the United Kingdom and further afield in Europe and the rest of the world. However, arguably the most significant effect of the BSE epidemic was the transmission of

BSE through diet to the human population with the consequent new, fatal disease, variant CJD. Variant CJD has been greatest in the United Kingdom, but has also been found worldwide. Measures that were put in place to prevent transmission of BSE to humans, between cattle and to other animals seem to have prevented the worst scenarios that were predicted when the disease was first described. However, there is still the potential for further cases in other genotypes and for onward transmission via, for example invasive medical procedures. Surveillance is imperative to monitor vCJD in humans and also to ensure that if other forms of TSEs, such as atypical scrapie or BASE in cattle, are spread to humans through diet, these are detected early in order for public health measures to be implemented.

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Chapter 6
Strategies for On-Line Decontamination of Carcasses

Oleksandr A. Byelashov and John N. Sofos

Introduction

Microbial food safety has been one of the most important challenges for the meat industry during the last two decades due to important foodborne outbreaks traced to contaminated products and associated costly product recalls from the market. *Escherichia coli* O157:H7 and other non-O157 Shiga toxin-producing (STEC) strains, as well as *Salmonella* serotypes, *Campylobacter jejuni*, *Clostridium perfringens, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolitica, Aeromonas hydrophila*, and *Bacillus cereus*are important pathogenic contaminants of meat and poultry products (Sofos, 2004a). STEC, especially, have been of major concern for the beef industry for a number of years, since for almost two decades contaminated beef products have been major sources of foodborne *E. coli* O157:H7 infection (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005).

In response to an outbreak (Bell et al., 1994) of *E. coli* O157:H7 infection traced to contaminated ground beef patties, the Food Safety Inspection Service of the United States Department of Agriculture (USDA-FSIS) implemented a "zero tolerance" rule for visible contamination on carcasses (USDA-FSIS, 1993). This requires knife trimming or steam vacuuming (for soil spots $\leq$ 2.5 cm in diameter) to remove physical contaminants such as bovine feces, ingesta, and udder fluids from the carcass surface before washing or spraying (USDA-FSIS, 1994). In addition, under the current United States federal regulation, *E. coli* O157:H7 is considered an adulterant in all non-intact beef products (USDA-FSIS, 1999a). To further improve the hygienic status of meat and poultry and increase the level of public health protection, USDA-FSIS

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changed its inspection regulation to require that slaughter plants develop and implement written Sanitation Standard Operating Procedures (SSOP), employ regular microbial testing, establish pathogen reduction performance standards, and develop and implement Hazard Analysis and Critical Control Point (HACCP) programs (USDA-FSIS, 1996b). Other countries or groups of countries, like the European Union, have also adopted the HACCP system and are considering potential use of antimicrobial interventions on carcasses. USDA-FSIS also recommends the use of decontamination interventions to reduce pathogen levels on carcasses prior to chilling (USDA-FSIS, 1996b). These regulations have led to development, testing, and implementation of antimicrobial intervention strategies to reduce or eliminate pathogens from meat and poultry products. According to a United States national survey, all United States large slaughter plants processing cattle, swine, lamb, goats, and other food animals (Cates, Viatoriatoriator, Karns, & Muth, 2008) and 98.4% of poultry slaughter facilities (Cates et al., 2006) employ at least some type of carcass decontamination techniques. Eighty one percent of these slaughter facilities used steam vacuuming to remove visible/microbiological contaminants, while approximately the same proportion of United States plants sprayed carcasses with organic acids, and about a half of all large plants used steam pasteurization of carcasses (Cates, Viatoriatoriator, Karns, & Muth, 2008).

This chapter covers strategies that are currently used by meat processors, or those evaluated under laboratory or/and in-plant conditions and available for decontamination of animals and animal carcasses at the various stages throughout the slaughtering process and carcass fabrication. The emphasis is placed on interventions applicable to, or used by the United States beef industry; however, most of the strategies discussed here are also applicable to other animal species, and may be used in the United States or other countries, depending on government regulations, common industry practices, and consumer acceptability of products. None of the decontamination strategies discussed in this chapter should be used as a sole intervention or to substitute good hygienic practices. Instead, many of these are used sequentially or in combination according to the multiple hurdle concept to eliminate or reduce pathogens to acceptable levels.

In discussing the effectiveness of antimicrobial interventions, we have emphasized effects on pathogens of public health concern, including *E. coli* O157:H7 and *Salmonella*. These pathogens are most commonly associated with food animals and raw meat products due to the inherited pattern of their distribution in nature. Also, we have discussed groups of fecal indicator bacteria such as *Enterobacteriaceae*, coliforms, and *E. coli*; presence and/or levels of these groups suggest potential contamination with pathogenic bacteria, which pose a health risk to consumers, if foods are improperly cooked or held under conditions supportive of bacterial growth.

Interventions Before Hide Removal

Cleaning of Live Animals

Food animals carry human pathogens in their gastro-intestinal (GI) tract and on their hides without displaying any physical or external symptoms (Hancock, Rice, Thomas, Dargatz, & Besser, 1997; Letellier, Messier, & Quessy, 1999; Vali et al., 2007). The hides or fleece of food animals are often soiled with feces, blood, dirt, and other foreign materials and are recognized as major sources of contamination in slaughter plants (Barkocy-Gallagher et al., 2003; Bell, 1997; Sheridan, 1998). Foodborne pathogens present on these materials may contaminate the sterile underlying muscle and fat tissue during slaughter through direct transfer or aerosols (Sofos, 2004b). Contamination is first introduced during the first knife incision for hide removal. Thus, this step should be considered as an important contamination transfer point during slaughter (Gill, Bryant, & Landers, 2003; Nou et al., 2003). In order to reduce pathogen levels transferred to carcasses and to the plant environment during hide removal, slaughter plants may implement various animal hide washing or cleaning strategies (Sofos, 2005) which may be applied before or after stunning (Fig. 6.1).

Clipping or hair trimming (Baird, Lucia, Acuff, Harris, & Savell, 2006; Small, Wells-Burr, & Buncic, 2005), spray washing with warm, cold, or ozonated water (Bosilevac, Shackelford, Brichta, & Koohmaraie, 2005), or antimicrobial solutions (Baird et al., 2006; Biss & Hathaway, 1995; Small et al., 2005), as well as bathing animals in a trough (Biss & Hathaway, 1995), may be used to reduce the levels of external hide contamination. Power-hosing animals for up to 10 min (Biss & Hathaway, 1995) with cold potable water (10–18°C) in cases of excessive soiling may significantly reduce levels of pathogenic bacteria (Byrne, Bolton, Sheridan, McDowell, & Blair, 2000). Spray washing with cold water seems to be especially effective in cleaning of small animals such as sheep or goats. Pre-stunning washing of sheep is routinely used in New Zealand regardless of contamination level, but in some instances it is applied to animals with extensive soiling of the pelt only (Biss & Hathaway, 1995, 1996; Byrne, Dunne, Lyng, & Bolton, 2007). United States federal regulations require that animals are not wet or at least not dripping at the time of slaughter (Reed, 1996). Therefore, washing should be performed with ample time to allow animals to dry off, or excessive water may be removed with blowing air or vacuum. Animal cleaning and washing, however, may cause stress when applied to live animals, which may be considered inhumane, and have undesirable effects on meat quality.

Live animals may be segregated and slaughtered in groups, based on degree of soiling. Processing speed of heavily soiled animals may be reduced, additional workers may be placed on the slaughter line, or other hygiene measures to reduce hide-to-carcass contamination may be employed (Biss & Hathaway, 1995; Byrne et al., 2007). This approach has been implemented in some

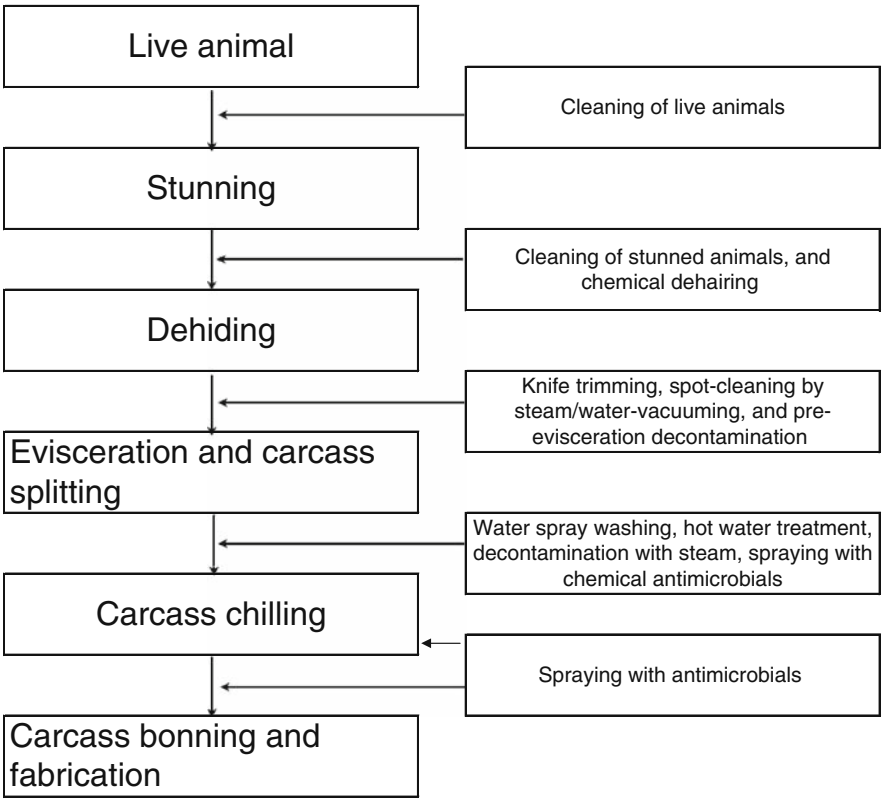

Fig. 6.1 Diagram of on-line decontamination strategies

European countries for cattle and sheep. For example, the Irish Department of Agriculture and Food requires a five-scale classification of cattle intended for slaughter. Based on their cleanliness and degree of dampness, they use colored tags to keep track of this classification during slaughter (Anonymous, 1997). However, a study conducted at a Finnish beef slaughter plant indicated that carcasses from excessively filthy animals, even when harvested with additional precautions, experienced higher microbial contamination levels compared to control carcasses fabricated from relatively clean animals (Ridell & Korkeala, 1993). Similarly, an Australian survey indicated that slaughter plants which are frequently processing soiled animals, on average, have a higher prevalence of *E. coli* on fabricated beef carcasses (Kiermeier et al., 2006). Perhaps excessively soiled animals need to be completely restricted from entering a slaughter facility (Ridell & Korkeala, 1993), or slaughtered at the end of the shift, but this approach may not be feasible due to facilities or economical limitations. In Australia, overnight misting of cattle in a holding pen with water containing detergent results in cleaned animal hides by the time of slaughter.

Cleaning of Stunned Animals

Stunned animals may be either spray washed with water or chemical solutions, dehaired, or treated with steam (McEvoy et al., 2003; McEvoy, Doherty, Sheridan, Blair, & McDowell, 2001; Sofos, 2005). Chemical solutions that are used or available for use by meat packing plants include quaternary ammonium compounds, surfactants, hydrogen peroxide, peracetic, lactic and acetic acid, ethanol, sodium hydroxide, acidified chlorine, cetylpyridinium chloride (CPC), and other antimicrobials, used individually or as ingredients in patented formulations (Baird et al., 2006; Bosilevac, Nou, Osborn, Allen, & Koohmaraie, 2005; Bosilevac, Nou, Barkocy-Gallagher, Arthur, & Koohmaraie, 2006; McEvoy et al., 2003, 2001; Mies et al., 2004; Small et al., 2005). It is recommended that a final rinse be used, followed by vacuuming, to improve the decontamination effects and to remove excessive liquid (Bosilevac, Nou, Osborn, Allen, & Koohmaraie, 2005). One of the major United States commercial beef processors employs hide-on pre-evisceration washing of carcasses in an on-line washing cabinet using sodium hydroxide as an antimicrobial (Koohmaraie et al., 2005). This intervention may allow almost a threefold reduction of *E. coli* O157 prevalence on hides (Koohmaraie et al., 2005). Some high-volume beef slaughter plants spray stunned animals with cold water in a spraying cabinet in order to moister animal hides and prevent the distribution of dust into the air. This step is important, as aerosol contamination may significantly compromise the microbiological quality of meat (Burfoot et al., 2006; Rahkio & Korkeala, 1997). Some packing plants minimize such contamination by optimizing plant design and process flow and by using an airflow system directed from less potentially contaminated areas, at the end of a processing line, toward more heavily contaminated operational sections of the plant. In addition, clipping beef hides followed by singeing or spray washing may effectively reduce contamination under laboratory conditions but the procedure is considered time-consuming, and therefore, may have no practical application in commercial-scale processing plants (Baird et al., 2006; Small et al., 2005; Sofos, 2006).

Chemical Dehairing

There exists a patented method for chemical removal of hair from animals after stunning (Bowling & Clayton, 1992; Schnell et al., 1995). This multi-step procedure involves application of a sodium sulfide solution, the depilatory chemical, followed by pressurized water spraying of animals to remove hydrolyzed hair, and then spraying with a neutralizing solution, sodium carbonate or sodium bicarbonate in combination with hydrogen peroxide, in a washing cabinet (Bowling & Clayton, 1992; Nou et al., 2003). However, sodium sulfide is rapidly converted into hydrogen sulfide, which is an environmental pollutant that may be toxic to humans (Gehring, Dudley, Mazenko, & Marmer, 2006;

Marmer & Dudley, 2004, 2005a, 2005b). As an alternative to this method, magnesium peroxide, potassium peroxymonosulfate, sodium percarbonate, alkaline hydrogen peroxide, and potassium cyanate have been evaluated and recommended for use (Gehring et al., 2006; Marmer & Dudley, 2004, 2005a, 2005b). Chemical dehairing was suggested for use to remove physical contamination and reduce microbial load before hide removal, and thus, to prevent introduction of contaminants in the main slaughter room (Bowling & Clayton, 1992). Schnell et al.(1995) first examined the effect of chemical dehairing of beef carcasses and reported that the process reduced visible contamination and amount of product trimmed by knife to comply with zero tolerance requirements, but did not affect levels of total aerobic bacteria and *E. coli*; however, the study was conducted during normal operation in a large beef slaughter facility and the dehaired carcasses were exposed to cross-contamination from conventional carcasses, workers, and aerosols. Later, Graves Delmore (1998) reported that this dehairing process substantially reduced numbers of total bacteria, coliforms, *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* on hide samples that were artificially contaminated with inoculated feces in a laboratory scale study, as well as numbers of total bacteria and coliforms on beef carcasses under in-plant conditions. Greater microbial reduction was achieved under the laboratory conditions, as in-plant dehaired carcasses were processed during normal plant operation together with non-dehaired carcasses (Graves Delmore, 1998). Similarly, Castillo, Dickson, Clayton, Lucia, and Acuff (1998) found that chemical dehairing significantly reduced numbers of aerobic bacteria, coliforms, *E. coli* (including O157:H7), and *S.* Typhimurium under laboratory conditions. Despite being an effective decontamination intervention, chemical dehairing of animals is not used by the United States beef industry at this time (Koohmaraie et al., 2005; Sofos, 2005). One of the disadvantages of this intervention is the cost associated with installation and operation of closed cabinets, and the reduced speed of the overall slaughtering process. Also, there are additional costs related to handling of chemicals before and after use as well as processing of generated waste which includes chemical pollutants and hydrolyzed animal hair.

Interventions During and After Hide Removal But Before Evisceration

Hide Removal

The carcass surface below the hide is initially sterile (Sofos, 1994). However, it becomes contaminated during hide removal. Dehiding is a multi-step process, which in high-volume United States operations is usually performed by several on-line workers and involves the following sequential steps: opening hides at rear hocks, removal of hoofs, skinning the butt, opening the brisket and tail

skin, skinning the brisket and back, and then pulling the hide using cattle skinning machines or "hide-pullers" (Gill, McGinnis, & Badoni, 1996). Most of these steps involve passage of a knife through heavily contaminated hide areas resulting in contamination of underlying tissues (Gill, McGinnis, et al., 1996). Therefore, all precautions should be taken to minimize exposure to hide contamination during hide removal, and to apply decontamination interventions, during following stages of slaughter, to remove or inactivate contamination that may have been introduced during this and other steps of the process.

Knife Trimming

As indicated, USDA-FSIS recommends that skinned carcasses be free of any visible contamination including fecal matter, ingesta, hair, abscesses, bruised tissues, and udder contents before application of any washing, spraying, and chilling (USDA-FSIS, 1993). According to United States federal regulations, physical contaminants of less than 1 in. (2.54 cm) in diameter may be removed using steam vacuuming or vacuuming with hot water (Kochevar, Sofos, Bolin, Reagan, & Smith, 1997; USDA-FSIS, 1996a), whereas larger contaminated areas need to be manually excised by knife trimming (USDA-FSIS, 1993).

Knife trimming is an extensively used commercial practice in high-speed United States animal slaughter plants. In addition to improvement in aesthetic appearance, knife trimming, when performed correctly, contributes to reduction of carcass microbial contamination as it removes portions of tissue that are likely to be heavily contaminated with bacteria (Ellerbroek, Wegener, & Arndt, 1993). This procedure is of particular importance in removal of contamination in areas that are difficult to access using washing or spraying (e.g., inside portion of the round, which lies on the inside of the leg of animal carcasses) (Hardin, Acuff, Lucia, Oman, & Savell, 1995). Knife trimming alone or knife trimming followed by spraying/washing have been shown to be effective in most published studies conducted under laboratory conditions using sterile utensils for removal of contamination from beef cuts (Gorman, Morgan, Sofos, & Smith, 1995; Gorman, Sofos, Morgan, Schmidt, & Smith, 1995) or carcasses (Castillo, Lucia, Goodson, Savell, & Acuff, 1998a; Graves Delmore, Sofos, Reagan, & Smith, 1997; Hardin et al., 1995; Phebus et al., 1997; Prasai et al., 1995; Schnell et al., 1995) as well as in large-scale slaughter plants (Reagan et al., 1996). Reagan et al.(1996) examined the effectiveness of knife trimming at six high-volume (100–400 carcasses per hour) beef slaughter facilities located in five different states and operated by four independent companies and reported that this intervention reduced levels of microbial contamination by 1.3 log CFU/cm^2. However, the effectiveness of the intervention depends on the skill, motivation, and carefulness of workers who visually detect contamination and trim carcasses (Reagan et al., 1996). Gill and Baker (1998) reported that knife trimming of lamb carcasses (as well as vacuum-cleaning) was not effective in

reducing total aerobic counts, coliforms, and *E. coli* and suggested use of these interventions for reduction of visible contamination only. Similarly, a separate study conducted in a high-volume beef packing plant (280 carcasses per hour) demonstrated that knife trimming was not effective in reduction of total microbial loads (Gill, Badoni, & Jones, 1996). Trimming, as well as other decontamination strategies, can result in accidental cross-contamination from heavily contaminated portions of a carcass to parts that are less contaminated (Castillo et al., 1998a; Schnell et al., 1995), or lead to spreading of bacteria among carcasses (Edwards & Fung, 2006). This is because areas contaminated with fecal bacteria may be larger than visibly contaminated spots (Edwards & Fung, 2006). Trimming is usually performed with personal skinning knives and hooks of plant employees (Gill & Ginnis, 2003; Reagan et al., 1996). If not consistently and properly cleaned, these tools can become heavily contaminated with pathogens (Smeltzer, Peel, Peel, & Collins, 1979). The cleaning and sanitizing of individual equipment are based on the worker's personal decision and may not be as adequate, effective and/or consistent as that of stationary plant equipment (Gill, Badoni, & McGinnes, 1999). Therefore, plant standard operating procedures (SOP) must include precise instructions for cleaning and sanitizing of personal tools used for trimming or for other processing operations (Prasai et al., 1995; Reagan et al., 1996). It may be impractical to decontaminate knives and hooks after every cut, especially in high-speed processing plants. However, it may be reasonable to decontaminate the equipment before the trimming of each new carcass, thereby at least preventing carcass-to-carcass cross-contamination (Edwards & Fung, 2006; Reagan et al., 1996). Sanitizing of knives and hooks can be done by immersion in hot (82°C) water or other sanitizers for a minimum of 15 s (Gill & Ginnis, 2003; Taormina & Dorsa, 2007). However, in high-volume slaughter plants, the immersion of personal equipment for 15 s was not practical because of the high speed of the processing line (Taormina & Dorsa, 2007). To avoid this problem, the high-volume packing plants implement the "double knife" system, where one of the knives is immersed in hot water, while the other is in use, with knives being frequently rotated. The use of this system can be optimized choosing an appropriate time–temperature combination (Goulter, Dykes, & Small, 2008).

Spot-Cleaning by Steam/Water Vacuuming

In 1996, the USDA-FSIS issued a notice of policy change which required using secondary decontamination strategies, including steam vacuuming in addition to, or instead of, knife trimming for spots less than 2.5 cm in diameter (USDA-FSIS, 1996a). This occurred as a result of an extensive testing of vacuuming systems in more than 50 commercial processing plants (USDA-FSIS, 1996a). Part of this testing was performed by researchers at the Department of Animal Sciences at Colorado State University and demonstrated the effectiveness of

steam vacuuming and the capability of different packing plants to control the intervention over time (USDA-FSIS, 1996a). Currently, steam vacuuming is widely used in United States animal slaughter facilities for spot-cleaning of visibly soiled ($\leq$2.5 cm) carcass areas or areas expected to carry microbial contamination (Sofos & Smith, 1998). The method includes application of steam or hot water which loosens up the soil as well as detaches and destroys or injures bacteria (Dorsa, Cutter, Siragusa, & Koohmaraie, 1996; Kochevar et al., 1997). Steam/hot water treatment is followed by vacuuming which removes physical and biological contaminants (Dorsa, Cutter, Siragusa, & Koohmaraie, 1996; Kochevar et. al., 1997). Typically, a commercial hand-held steam vacuuming unit includes a stainless steel vacuum head which pulls a vacuum (usually about –0.0093 bar), as it directly contacts the carcass surface. This head is fitted with an inside nozzle that sprays hot water (>82°C at 0.34–1.03 bar); alternatively, a hand wand that ejects steam (82°C) may be used instead of a nozzle. In addition, the vacuum head is continuously sanitized from the outside with hot steam (Kochevar et al., 1997). The vacuum may be applied to an area with vertical motions for 5–10 s; filth that is loosened from a carcass surface is drawn by the vacuum into a waste collecting tank. Similar to knife trimming, the effectiveness of this process varies depending on the personal judgment and proficiency of the equipment operator, as well as the working condition of the equipment (Kochevar et al., 1997; Sofos & Smith, 1998), and, therefore, must be performed by trained individuals, using well-functioning equipment.

Kochevar et al. (1997) reported that the extent of removal of visual and microbial contamination from beef carcasses using steam vacuuming was similar to that obtained by knife trimming. In that study steam vacuuming (82°C; –0.0093 bar) was performed in five commercial beef-slaughtering and dressing facilities by plant employees and reduced aerobic plate counts (APC) and total coliforms on carcasses by 1.7–2.2 log CFU/cm^2. Studies conducted under laboratory conditions displayed even greater microbial reductions. Dorsa, Cutter, and Siragusa (1996) evaluated the effect of steam vacuuming on microbial reduction on beef carcass short plate primal cuts that were artificially contaminated with a fresh cattle fecal slurry. A hand-held system delivered a combination of water and steam (88–94°C) followed by vacuuming (–0.3386 bar). The researchers demonstrated that this intervention reduced APC, total coliforms and *E. coli* by 3–4 log-cycles and indicated that steam vacuuming may cause a temporary discoloration of treated areas of the meat (Dorsa, Cutter, & Siragusa, 1996). Another study conducted by the same group demonstrated that the use of the same equipment delivered a 5.5 log CFU/cm^2 reduction of *E. coli* O157:H7 on beef carcass short plates inoculated with 7.6 log CFU/cm^2 of the pathogen (Dorsa, Cutter, & Siragusa, 1997a). A separate study indicated that using the same apparatus may reduce populations of APC, *L. innocua*, and lactic acid bacteria by 1.6–2.0 log CFU/cm^2 (Dorsa, Cutter, & Siragusa, 1997b). However, bacteria resumed growth after 2 days of storage at 5°C and increased by approximately 3 log-cycles by day seven (Dorsa et al., 1997b). This observation indicated the need for application of additional

interventions that convey bactericidal effects. Contrary to these reports, Gill and Bryant (Gill & Bryant, 1997) showed that hot water vacuuming resulted in non-significant, less than one-log reduction of APC, coliforms, and *E. coli* on beef carcasses when used at a high-volume (280 heads per hour) beef slaughter plant. Overall, despite some discrepancies in results, most published studies demonstrated the effectiveness of steam vacuuming in reducing visible and microbial contamination. Therefore, this technology is widely used in United States meat-processing plants today as it reduces the need for manual trimming of visible contamination.

In the United States, use of steam-/water vacuuming systems in the meat industry is regulated by the USDA-FSIS. It is required that the system works without significant temperature fluctuation and shuts off automatically if the temperature of steam/water at the carcass surface drops below 82.2°C (USDA-FSIS, 1996b). In addition, the vacuum pressure must be adequate to remove any residual water from the sprayed area, the vacuum head surface should be continuously sanitized with hot water or steam, and its temperature should be maintained at 82.2°C or higher (USDA-FSIS, 1996b).

Preevisceration Decontamination

Presence of pathogens on carcass surfaces after skinning is not restricted only to areas of visible contamination. In addition to animal and hide sources, bacteria may be introduced from processing equipment, hands of employees, aerosols, or other vectors at any step of carcass handling. Bell, Cutter, and Sumner (1997) reported that hands of plant employees that directly contact animal hides have bacterial contamination levels similar to the hide themselves, and, therefore, may serve as a significant source of carcass contamination. Therefore, United States beef-slaughtering operations, in addition to knife trimming or steam vacuuming of visible spot contamination, often apply spray washing/decontaminating processes of whole carcasses prior to evisceration (Bosilevac et al., 2006; USDA-FSIS, 1996c). These are considered as the most effective and practical sequence of interventions (Prasai et al., 1995). Washing/spraying carcasses, that do not meet "zero tolerance" inspection requirements for visible contamination, is prohibited by law (USDA-FSIS, 1996b). However, it may be applied before and/or after removal of viscera as a follow-up to knife trimming to reduce microbial contamination. Given that the strength of microbial attachment increases with time, spray washing immediately or shortly after a potential contamination step may be of particular importance (Dickson, 1995). Further, such intervention changes carcass surface properties, decreases the ability of soil and bacteria to attach, and, therefore reduce the susceptibility to further contamination, improving the efficacy of post-evisceration decontamination steps (Dickson, 1995). Currently, many United States slaughter plants use warm (approximately 42°C) 2% lactic acid in on-line spray cabinets as a

pre-evisceration wash (Bosilevac et al., 2006), while some establishments have adopted spray washing with hot water (74°C) (Bosilevac et al., 2006). Bosilevac et al. (2006) compared the effects of hot water washing (74°C, 5.5 s, 48.3 bar) to 2% lactic acid spraying at 42°C applied on beef carcasses before evisceration in industrial washing cabinets (Chad Co., Olathe, Kans.) in a large (380 head per hour) meat packing plant. It was reported that hot water washing reduced the prevalence of *E. coli* O157:H7 by 81%, which represented a significant reduction compared to that (35%) obtained by lactic acid alone (Bosilevac et al., 2006). In addition, hot water washing reduced APC and *Enterobacteriaceae* levels by 2.7 log-cycles, which was greater than reductions (1.0–1.6 log CFU/cm^2) achieved by lactic acid (Bosilevac et al., 2006). Therefore, it was suggested that use of hot water may be a more effective pre-evisceration intervention, compared to warm lactic acid. However, this conclusion must be interpreted with caution, as it is a well-established fact that organic acids have a delayed or bactericidal effect, restricting microbial growth (Ikeda, Samelis, Kendall, Smith, & Sofos, 2003; Koutsoumanis et al., 2004; Smulders & Woolthuis, 1985) and, in some instances, result in further reduction of microbial levels as injured cells may die off during refrigerated storage in the acidified environment (Greer & Dilts, 1995). Further, use of hot water may be limited on some plants due to condensation problems.

Interventions During and After Evisceration

Bung Tying and Evisceration

Bung (perianal region) tying is a part of the evisceration process. When combined with the tying of the esophagus or weasand (performed immediately after animal bleeding and exsanguination by a cut through the neck) the procedure prevents leakage of the rumen and fecal matter on carcasses and into the processing environment, thus, reducing spreading of bacteria (USDA-FSIS, 2002). The bung tying operation involves manual incision of the skin which surrounds the rectum, pulling the bung and covering it with a plastic bag (McDowell, Sheridan, & Bolton, 2005). This procedure must be performed in a manner that minimizes contamination from the anus area to the carcass surface via employees' hands and utensils or reduces cross-contamination between carcasses. It was reported that manual bung tying improved microbial quality of carcasses in commercial sheep slaughter operations (Hudson, Mead, & Hinton, 1998). Bung tying can be also performed using a commercially available automated system (Sheridan, 1998). The system was tested under in-plant conditions, demonstrating that total microbial, coliform, and *E. coli* counts were lower on carcasses processed using the automated system, compared to those processed using the manual bung tying method (Sheridan, 1998).

As indicated previously, the GI tract contains large numbers of bacteria, including the pathogens of public health concern. Therefore, evisceration is a

step that has a high potential for the spread of contamination. Generally, evisceration is performed within the first 30 min after bleeding, as stomach and intestines bulge with time elapsed after exsanguination, making it difficult to remove viscera and increasing the possibility of paunch and intestinal rupturing (Edwards & Fung, 2006). Due to high risk of carcass contamination with GI tract contents, evisceration should be performed by trained plant personnel and with special caution. The personnel should be also trained to perform corrective actions, which need to be done in case of carcass contamination due to rupture, puncture, or cutting of the viscera. These corrective actions include the removal of visible contamination by knife trimming, spray washing, and water/steam vacuuming of contaminated areas (USDA-FSIS, 2002). Removed viscera then undergoes the animal pathology evaluation by inspectors (Edwards & Fung, 2006).

Carcass Splitting

Bruises and damaged tissues must be removed from the midline area of the back of eviscerated carcasses to prevent contamination of underlying tissues during carcass splitting (USDA-FSIS, 2002). Carcass splitting is performed with a saw or cleaver along the vertical midline and is followed by the spinal cord removal, as required by regulation, with a knife and a hook, or using an automated vacuum system (McDowell et al., 2005).

Water Spray Washing

Carcass splitting is followed by spray washing with cold water to remove bone dust and blood from the carcass surface. Spraying/washing of animal carcasses with water (at temperatures that do not injure or kill bacteria) has been extensively researched, and on average, provides approximately a 90% physical reduction of microbial populations (Siragusa, 1995). Phebus et al. (1997) reported that washing of freshly slaughtered beef artificially inoculated with *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* with water (35°C, 23 s, 2.6–2.8 bar) reduced the pathogen numbers by 0.7–1.3 log-cycles. Similarly, Gorman, Sofos, et al. (1995) demonstrated that spray washing (35°C, 12–36 s, 2.8–27.6 bar) of adipose samples inoculated with feces containing *E. coli* ATCC 11370 significantly reduced microbial levels without redistribution of contamination to adjacent areas; greater reductions were obtained at higher spraying pressures. In a separate study, the same researchers evaluated the effect of hand trimming and various spraying solutions (including plain cold or warm water) in a model spray washing cabinet on beef brisket fat samples inoculated with *E. coli* ATCC 11370 and reported no redistribution of bacteria to the adjacent areas (Gorman, Sofos, et al., 1995). Contrary to these findings, it

was reported that spray washing lamb carcasses with cold (12°C) water for 20 s resulted in *Salmonella* contamination of the dorsal area, a part of the carcass that is less likely to be soiled during hide removal, whereas contamination levels of the more soiled, ventral area, remained unchanged (Ellerbroek et al., 1993). Similarly, there was no reduction in *E. coli* contamination when dehided beef carcasses were spray washed with cold potable water (Bell, 1997). The effectiveness of spray washing with cold water alone received some criticism, as it may result in redistribution of contamination (Ellerbroek et al., 1993), because some parts of carcasses may carry substantially higher microbial loads, as compared to others (Roberts, 1980). In addition, as spray washing with plain water may increase amount of moisture on carcass surfaces, there is a concern that this may also increase the rates of pathogen proliferation during potential temperature abuse (Ellerbroek et al., 1993). However, the effectiveness of spray washing depends on multiple factors, including water temperature, pressure, duration of spraying, and type of nozzle and spraying equipment (Sofos & Smith, 1998). Therefore, to restrict pathogen growth during storage and to decrease microbiological problems associated with redistribution of bacteria, spray washing with water alone must be followed by treatment with antimicrobials or hot water (Sofos & Smith, 1998).

Hot Water Treatment

In contrast to cold or warm water treatments, hot water ($\geq$ 74°C) appears to be more effective in reducing carcass microbial loads as it injures or kills bacteria (Cabedo, Sofos, & Smith, 1996; Hardin et al., 1995). Australian monitoring data indicated that beef slaughter plants using hot water decontamination systems, on average, had lower prevalence of *E. coli* on dehided carcasses (Kiermeier et al., 2006). One of the first studies on the effect of hot water on microbial quality of animal carcasses indicated that immersion of sheep carcasses in hot water (80°C for 10 s) destroyed 99% of coliforms and 96% of total aerobic bacteria (Smith & Graham, 1978). Later, Australian researchers designed an industrial hot water washing cabinet for decontamination of beef carcass sides (Davey, 1989; Davey & Smith, 1989). This equipment cascaded a wall of hot water from low-pressure nozzles and was simple and relatively inexpensive in installation and operation (Davey, 1989; Davey & Smith, 1989). The researchers tested a range of temperatures (44.5–83.5°C) for 10 or 20 s and reported that washing carcasses for 10 s at 83.5°C reduced counts of *E. coli* by 2.2 log-cycles, without having any permanent negative effect on carcass appearance (Davey & Smith, 1989).

It was demonstrated in a pilot plant study that spraying carcasses with hot water (95°C) for 10 s increased carcass surface temperature to 82°C and provided 1.3 log CFU/cm^2 reduction of APC (Barkate, Acuff, Lucia, & Hale, 1993). This intervention caused a slight visual discoloration of the carcass

surface immediately after treatment; however, the normal carcass color was restored after 24 h of refrigerated storage (Barkate et al., 1993). The authors also indicated the need for designing appropriate washing equipment (Barkate et al., 1993). Later, Gill, McGinnis, Bryant, and Chabot (1995) tested wash-pasteurization equipment fitted with a water recirculation system for decontamination of polished, uneviscerated pig carcasses in a commercial processing plant. Sheets of hot water were delivered in an industrial scale cabinet at temperatures between 60 and 90°C for 20–90 s (Gill et al., 1995). Upon examination of 800 treated carcasses, water washing at 85°C for 20 s was recommended for on-line carcass decontamination as it reduced populations of *E. coli* by a maximum of 2.5 log-cycles (Gill et al., 1995). Following this research, a full-scale commercial cabinet was patented, installed, and tested over a 3-month period in a pig slaughter plant (Gill, Bedard, & Jones, 1997). This equipment was capable of pasteurizing 1,200 pig carcasses per hour with each carcass subjected to hot water (85°C) for 15 s, proving the efficiency and practicality of this cabinet in high-volume slaughter plants (Gill et al., 1997). Further, it was demonstrated that washing eviscerated pig carcasses or half-carcasses (85°C, 10 s) as well as skinned sheep carcasses (83°C, 18 s) using a water-pasteurizing apparatus reduced numbers of coliforms and *E. coli* by more than 2 log-cycles, while APC were reduced by more than 1 log-unit (Gill, Jones, & Badoni, 1998). This treatment did not affect the overall appearance of animal carcasses, but did cause slight discoloration of cut muscle surfaces (Gill et al., 1998). Similarly, in a separate study, hot water pasteurizing (85°C, 10 s) of beef carcass sides reduced APC and *E. coli* by 1.5 and 2.0 log CFU/cm^2, respectively, without any negative effect on appearance of the product (Gill, Bryant, & Bedard, 1999).

Castillo, Lucia, Goodson, Savell, and Acuff (1998b) tested a two-step hot water washing procedure against visible fecal contamination, and spoilage and pathogenic bacteria on beef carcasses contaminated with inoculated bovine feces. The initial step of the process included a low-pressure washing (25°C, 0.7 bar, 90 s) with a hand-held apparatus followed by a high-pressure washing in a spray cabinet (Chad Co., Lenexa, Kans.) that delivered warm (35°C) water for 9 s at variable pressure (17.2–31.0 bar); this step was followed by the hot water washing (95°C, 1.7 bar, 5 s). The scientists reported that this procedure reduced *E. coli* O157:H7, *S.* Typhimurium, APC, and thermotolerant coliform counts by 3.7, 3.8, 2.9, and 3.3 log CFU/cm^2, respectively (Castillo et al., 1998b). Perhaps this procedure can be used for removing visible contamination as well as for improving microbiological quality; however, washing carcasses to remove visible contaminants is not allowed in the United States.

Overall, hot water washing/spraying/rinsing of carcasses has been extensively researched and its effectiveness is well established. Depending on pressure, exposure time, type and initial levels of bacteria, and other factors, this intervention delivers 1–3 log-unit reductions (Sofos & Smith, 1998). Other advantages of this intervention include the potential for equipment installation at various steps between the stunning of animals and the fabrication of primal/retail cuts; the possibility of addition of antimicrobials in the water to achieve

even greater microbial reductions; and the availability of commercial washing cabinets. Some disadvantages of this type of intervention may include accumulation of condensation, potential risk of scalding of plant workers, and temporary discoloration of treated meat surfaces.

Decontamination with Steam

Decontamination with pressurized steam, or "steam pasteurization", is one of the most effective interventions approved by the USDA-FSIS and is widely used in United States slaughtering plants (USDA-FSIS, 1996a). This patented and completely automated method (Willson, 1994) was tested in a laboratory scale (Phebus et al., 1997; Retzlaff et al., 2004) and in high-volume commercial beef slaughter facilities (Nutsch et al., 1997; Retzlaff, Phebus, Kastner, & Marsden, 2005). The method involves the following steps: removal of water from carcass side surfaces, which remains after post-evisceration washing, using air blowers or vacuum (this step is needed as residual cold water may protect the bacteria); surface "pasteurization" with pressurized steam (6.5–10 s); and a cold water spray to cool down carcass surfaces (Retzlaff et al., 2004). Nutsch et al. (1997) evaluated the effectiveness of patented steam pasteurizing equipment (8-s steam exposure, 90.5–94.0°C; Frigoscandia Food Processing Systems, Bellevue, Wash.) in a commercial slaughter facility with a processing speed of 240 animals per hour. The results indicated that the process significantly reduced APC by 1.3 log CFU/cm^2 and lowered the initial prevalence of *E. coli* (16.4%), total coliforms (37.9%), and *Enterobacteriaceae* (46.4%) to 0, 1.4, and 2.9%, respectively (Nutsch et al., 1997). Similarly, Retzlaff et al. (2005) reported that the Frigoscandia™ steam pasteurization system (85.0–87.8 °C; 10.5–11 s), which was installed on a high-volume processing plant (392 heads per hour), reduced initial populations of total coliforms, *Enterobacteriaceae*, and *E. coli* on carcasses to undetectable levels. This intervention was suggested as a critical control point (CCP), with a critical limit of 85°C as a minimum chamber temperature (Retzlaff et al., 2005). Another study evaluated the effectiveness of a British pasteurizing system (10 s exposure time; 90°C), using non-pressurized steam, in a smaller beef processing operation (60 heads per hour) and reported significant reductions of *Enterobacteriaceae* and *E. coli* on carcasses, whereas reductions of total APC were inconsistent, suggesting that the process should be used as an aid to good hygienic processing, rather than a CCP (Minihan, Whyte, O'Mahony, & Collins, 2003). A Canadian study demonstrated that the Frigoscandia™ system (105°C, 6.5 s), which was installed in a high-volume beef processing plant (280 animals per hour), decreased APC by about 1 log-cycle, whereas total coliforms and *E. coli* were reduced by more than 2 log-cycles (Gill & Bryant, 1997).

Installation of a "steam pasteurizing" unit requires large capital investment, equipment maintenance, and operating costs, but it has lower water and energy

consumption compared to hot water treatment (Sofos, Belk, & Smith, 1999). Therefore, treatment of animal carcasses with pressurized steam may be an additional, and possibly the final before chilling, step toward elimination or reduction of pathogens to an acceptable level (Sofos et al., 1999). However, similar to other interventions, the effectiveness of this strategy will depend on other hygienic measures used in processing facilities and the diligence of plant personnel who operate the equipment (Sofos et al., 1999).

Spraying with Chemical Antimicrobials

While spraying with water physically removes bacteria and depending on water temperature may cause thermal destruction/injury to cells, spraying with chemical antimicrobials, in addition to an immediate pathogen reduction, may also prevent or inhibit growth of surviving pathogens during storage. Chemical spraying may be included as a CCP in HACCP plans if validated for its efficacy (Dormedy, Brashears, Cutter, & Burson, 2000; Gill et al., 2003). Spray washing carcasses with antimicrobials is widely used by the meat-processing industry as it has been extensively researched in the laboratory and under in-plant conditions (Cutter & Siragusa, 1994a, 1994b; Delmore et al., 2000; Dormedy et al., 2000; Gill & Badoni, 2004; Gill et al., 2003; Koutsoumanis et al., 2004; Smulders & Woolthuis, 1985).

Chlorine

One of the first compounds investigated for decontamination of carcasses was chlorine (Cutter & Siragusa, 1995; Emswiler, Kotula, & Rough, 1976; Kotula, Lusby, Crouse, & Devries, 1974). Kotula et al. (1974) evaluated the effect of spraying pressure, temperature, and pH of the chlorine solution of 200 ppm on total aerobic bacteria on beef carcasses. The results indicated that the treatment was more effective at a high pressure (24.1 bar), at 51.1°C, and in the pH range of 6–7. The authors also reported that the magnitude of the reduction was greater when carcasses were tested within 24 h after treatment, compared to testing after 45 min. This is possibly due to the inability of injured cells to recover on the carcass surface when stored at a low refrigeration temperature (1.1°C) (Kotula et al., 1974). When averaged across pH and pressures tested, the reductions were 2.2 and 3.2 log CFU/cm^2 at 13 and 52°C, respectively. Another study (Emswiler et al., 1976) demonstrated that chlorine may be effective in reducing APC when applied at a concentration of at least 100 ppm (6.2 bar; pH 6.5; 12°C, 60 s). It was also reported (Northcutt, Smith, Musgrove, Ingram, & Hinton, 2005) that adding up to 50 ppm chlorine into water did not enhance the reduction of total aerobic bacteria, *E. coli*, or *Campylobacter* when broiler carcasses were sprayed (5.5 bar; pH 8; 5 s) at different temperatures (21–54°C).

Chlorine becomes inactive as it reacts with organic matter. Therefore, its effectiveness is limited to the free available chlorine in the solution (i.e., concentration of hypochlorous acid (HOCl) and hypochlorite ions (OCl) existing in chlorinated water) (Kotula, Kotula, Rose, Pierson, & Camp, 1997). The degree of deactivation depends on the amount and type of organic material present (i.e., it becomes more easily deactivated by lean muscle tissue compared to adipose tissue) (Kotula et al., 1997). Therefore, the experimental results on use of chlorine may vary considerably, depending on species of animals used for food, degree of fatness, and other experimental conditions. Stevenson, Merkel, and Lee (1978) reported no effect of a 200 ppm chlorine spray on APC on beef carcasses. Further, Cutter and Siragusa (1995) reported that even at higher concentrations (up to 500 ppm) chlorine was not significantly more effective than water in reduction of *E. coli* O157:H7 on beef.

The USDA-FSIS has approved the use of chlorine at the concentration of 20 ppm in poultry washes/sprays, and at 50 ppm in poultry chill tanks, but it is currently not permitted for decontamination of red meat carcasses (USDA-FSIS, 1995). However, there are some potential drawbacks to chlorine use in slaughter plants. The chemical is corrosive and can damage the equipment if used over an extended period of time (Eker &Yuksel, 2005; Sofos & Smith, 1998). In addition, there are some concerns that chlorine may form substances toxic to human, trihalomethanes, when it reacts with organic matter (Boorman et al., 1999; Richardson, 2003; Sofos & Smith, 1998). Further, inhalation of chlorine may be harmful to plant workers. Therefore, the meat packing industry presently uses mostly organic acids for carcass decontamination as they are not toxic and have other advantages over chlorine and other chemicals.

Chlorine Dioxide

Chlorine dioxide is an oxide of chlorine which exists as a gas under normal atmospheric conditions and at temperatures above 11°C. It may be a more suitable decontaminating compound for the meat industry, as it has a better oxidizing power and antimicrobial activity (Andrews, Keys, Martin, Grodner, & Park, 2002), is active at high pH, and does not react with organic matter (Sen, Owusuyaw, Wheeler, & Wei, 1989; Wei et al., 1987). There exists a patented method for spray chilling and decontamination of pork carcasses with an aqueous solution of 5–25 ppm chlorine dioxide (Svoboda & Schwerdt, 1977). However, Cutter and Dorsa (1995) reported that chlorine dioxide (0–20 ppm, 16°C) sprayed (5.2–6.9 bar, 10–60 s) in a pilot scale carcass washer did not reduce APC on beef carcass tissues inoculated with bovine feces, compared to samples treated with water only. Another study showed small, but significantly different (from those caused by plane water) microbial reductions, when chlorine dioxide (100 ppm) was used as a dip (4°C, 10 min) for chicken breasts and drumsticks inoculated with *E. coli* O157:H7 and *S.* Typhimurium (Yunhee et al., 2008).

The chemical is currently approved by the USDA-FSIS as a decontaminant of beef carcasses, parts and organs, and as an antimicrobial for water in poultry processing; the residual levels of the chemical should not exceed 3 ppm (USDA-FSIS, 2008). Potential drawbacks in the use of the chemical include the need for gas-generating system installation, difficulties in maintaining the target concentrations, and degradation of the compound during storage (Edwards & Fung, 2006; Shin, Chang, & Kang, 2004). Shin et al. (2004) proposed the use of ice-containing chlorine dioxide as an antimicrobial intervention against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* inoculated on the surface of fish. When antimicrobial ice (100 ppm ClO_2) was applied (120 min) to fish skin, numbers of the pathogens were reduced by 4.8, 2.6, and 3.3 log-cycles, respectively (Shin et al., 2004). This approach in chlorine dioxide application may be potentially used for decontamination of whole poultry carcasses or fabricated primal and subprimal red meat cuts.

Organic Acids

Lactic and acetic acids are widely accepted chemicals for carcass decontamination in the United States as they are generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA) (FDA, 2008) and are included in the USDA-FSIS list of safe and suitable ingredients for use in the production of meat and poultry products (USDA-FSIS, 1996a, 2008). They are relatively inexpensive and are permitted in the United States for use in carcass washing at levels of up to 2.5% for acetic acid (applied pre-chill) and at up to 5% for lactic acid (applied either pre- or post-chill) (USDA-FSIS, 2008). The bactericidal and bacteriostatic effect of lactic and acetic acids, individually or in combination, as a single intervention or as a follow up to other antimicrobial interventions was demonstrated in numerous studies (Cabedo et al., 1996; Cutter & Siragusa, 1994b; Delmore et al., 1998, 2000; Gill & Badoni, 2004; Koutsoumanis et al., 2004; Smulders & Woolthuis, 1985). Other organic acids including polylactic (Allanson, Curry, Unklesbay, Iannotti, & Ellersieck, 2000; Lim & Mustapha, 2003), gluconic (Zepeda et al., 1994), fumaric (Podolak, Zayas, Kastner, & Fung, 1996), peroxyacetic (Gill & Badoni, 2004), and citric (Cutter & Siragusa, 1994b) were studied less comprehensively, but they may also be used in the meat industry. The antimicrobial mode of action involves the damaging of cell membranes (Stratford & Anslow, 1998), altering of cell permeability (Alakomi et al., 2000), disruption of proton-motive force (PMF) (Eklund, 1985), and inhibition of metabolic reactions (Russell, 1992).

Reported reductions in bacterial populations on carcasses and meat and poultry surfaces caused by organic acids vary considerably as a function of spraying time, pressure, and temperature (Anderson et al., 1987; Anderson & Marshall, 1989), whereas the type of acid does not appear to be a major factor (Cutter & Siragusa, 1994b). Cutter and Siragusa (1994b) evaluated the effect of lactic, acetic, and citric acids (1, 3, and 5%; 24°C) as spraying solutions (5.5 bar) of beef tissue inoculated with *E. coli* O157:H7. Mean reductions for these acids,

averaged across concentration, ranged between 1.7 and 1.9 log CFU/cm^2 with no difference among acid types (Cutter & Siragusa, 1994b). Contrary to these findings, it was reported that lactic acid alone or as a mixture containing 2/3 lactic plus 1/3 acetic acid, applied at different temperatures, delivered a better bactericidal effect on lean beef inoculated with *E. coli* and *Salmonella* compared to acetic acid alone (Anderson, Marshall, & Dickson, 1992). Overall, the microbial reductions increased with temperature (from 20 to 70°C) of dipping solutions (Anderson, et al., 1992). Similarly, it was reported that reduction of populations of *Enterobacteriaceae*, *E. coli*, and *S.* Typhimurium increased with temperature when beef cuts were dipped in 1, 2, or 3% lactic acid. Other studies reported remarkable reductions of *E. coli* and APC when 4% lactic acid was applied at 55°C to beef carcasses under commercial plant conditions (Castillo, Lucia, Mercado, & Acuff, 2001).

Most of the skinned carcass surface is normally covered with fat. While it is clear that the magnitude of pathogen injury/destruction depends on the concentration and temperature of acids, the effect of the type of contaminated carcass tissue is not consistent throughout the literature. Cutter and Siragusa (1994b) reported higher efficacy of organic acids against *E. coli* O157:H7 on adipose compared to lean tissue samples. Contrary to these findings, Dickson (1992) reported that *S.* Typhimurium was reduced to a lesser extent on fat tissue, contaminated with manure-diluted inoculum compared to reduction on the lean tissue. Nevertheless, antimicrobial activity of organic acids applied to contaminated adipose tissue is well documented (Gorman, Morgan, et al., 1995; Gorman, Sofos, et al., 1995).

Differences in the study designs, types, temperature, and concentrations of acids as well as other experimental conditions, such as exposure time, and point of on-line application makes it difficult to compare acids in terms of their effect on microbial safety. However, within the range of concentrations permitted for use by the USDA-FSIS, organic acids may deliver a bactericidal and bacteriostatic effect in addition to the physical removal achieved by water/spray washing alone. One of the potential disadvantages of acid use is an accelerated corrosion of plant equipment (Eker & Yuksel, 2005) and temporary or permanent discoloration (which may or may not take place depending on the concentration, duration, temperature of application, type of tissues and other factors) of animal or poultry carcasses. In addition, concerns exist over the selection of acid-tolerant pathogens which may persist in the processing environment and acquire cross-protection to other stresses including chemical sanitizers of plant equipment (Sofos, 2002; Samelis & Sofos, 2003).

Other Chemical Antimicrobials

To date, organic acids remain one of the most frequently used compounds for decontamination of meat carcasses, whereas chlorine is most commonly used in poultry processing. Some other chemicals that may find some use and have been evaluated for their efficacy and proposed for use and/or approved by the United

States federal agencies include trisodium phosphate (TSP) (Cabedo et al., 1996; Dorsa et al., 1997a; Morris, Lucia, Savell, & Acuff, 1997; Okolocha & Ellerbroek, 2005), peroxyacid preparations, acidified sodium chlorite (ASC) (Castillo, Lucia, Kemp, & Acuff, 1999; Gill & Badoni, 2004), sodium lactate (Zeitoun & Debevere, 1992), CPC (Stopforth et al., 2004; Ransom et al., 2003), hydrogen peroxide (Bell et al., 1997; Cabedo et al., 1996; Mulder, Vanderhulst, & Bolder, 1987), potassium sorbate (Zeitoun & Debevere, 1990), sodium bicarbonate (Bell et al., 1997), electrolyzed oxidizing water (Fabrizio & Cutter, 2004; Kalchayanand et al., 2008), ozonated water (Kalchayanand et al., 2008), and nisin (Cutter & Siragusa, 1994a). Some of these are not extensively used in industry, because of the economical reasons, lack of approval by federal agencies, or low efficacy. However, CPC and TSP antimicrobials are successfully used as a pre- and post-chill spray in some poultry processing facilities (Beers et al., 2006; USDA-FSIS, 2008), and ASC and peroxyacid are used as a spray or dip for meat or poultry carcasses and parts (USDA-FSIS, 2008).

Other Decontamination Processes

Other processes that have been proposed as potential decontamination interventions, alone or in combination, include irradiation (gamma or electron beam), ultraviolet light, high hydrostatic pressure, infrared technology, electromagnetic fields, pulsed light, sonication, microwaves, bacteriophages, and bacteriocin-producing bacteria (Sofos, 2008; Sofos & Smith, 1998). Among these, ionizing irradiation is the most suitable and promising technology which involves discharge and translocation of energy in the form of waves or particles through space or a food without inducing radioactivity. The process destroys or injures microorganisms directly (by damaging bacterial DNA) or indirectly (via free radicals that are formed during water radiolysis) (Jay, 2005). In the United States, irradiation of poultry was approved by the FDA in 1990 (FDA, 1990) and by the USDA-FSIS in 1992 (USDA-FSIS, 1992). The irradiation of red meats is approved by the USDA-FSIS since 1999 (USDA-FSIS, 1999b). A logical point of application of irradiation in a slaughter plant could be immediately after chilling and before deboning, as application at earlier stages would leave an opportunity for carcass contamination during subsequent handling and chilling (Vosough-Ahmadi, Velthuis, Hogeveen, & Huirne, 2006). It was estimated that irradiation of beef carcasses after chilling is the only currently available intervention that would allow for more than 99% reduction of *E. coli* O157:H7 prevalence, compared to processing with no decontamination processes (Vosough-Ahmadi et al., 2006). Irradiation, however, is used by only 0.5% of all United States meat slaughter plants, and is applied after fabrication, and exclusively by very small processors (Cates et al., 2008). The most significant obstacles in the wider application of carcass irradiation in the United States are cost, lack of facilities, and low consumer demand due to limited acceptance of irradiated meats.

Carcass Chilling

If carcasses are not rapidly chilled, their temperature may increase to approximately 40°C due to pre-rigor muscle metabolism, and thus, provide the opportunity for microbial proliferation on carcass surfaces (Gill, 1995). Therefore, to prevent microbial growth, carcasses should be either boned out of their skeletons before rigor or chilled rapidly. The advantages of carcass boning shortly (approximately 45 min) after exsanguination, or "hot boning," over the conventional post-rigor primal/subprimal cuts fabrication are (i) faster chilling rates of primal cuts, compared to those of whole carcasses, due to the increased surface area and decreased mass; (ii) lower energy consumption; (iii) decreased manual labor; and (iv) decreased weight/moisture losses of cuts, if they are vacuum-packaged shortly after the fabrication (Rotterud et al., 2006). However, this approach creates certain concerns due to the increased handling of unchilled cuts by plant employees (which may potentially increase chances of contamination and subsequent microbial growth). In addition, there is a need for the rapid packaging of the cuts to prevent weight/moisture loss due surface desiccation. Further, hot boning may have a negative effect on meat tenderness (Tornberg, 1996). Therefore, most of the large-scale beef-slaughtering facilities in the United States fabricate carcasses after chilling.

Conventional chilling of beef carcasses generally takes about 2 days and involves the use of circulating cold air, which causes drying of carcass surfaces and weight/moisture loss, and therefore, is not desirable from an economical perspective (Kinsella et al., 2006; Mallikarjunan & Mittal, 1995). Thus, to prevent weight/moisture losses, increase chilling rates, and improve microbial quality, most major North American slaughter plants have adopted the spray chilling of carcasses prior to fabrication. Unites States federal regulations, however, require that meat and poultry carcasses do not gain weight as a result of spraying and water retention; otherwise, establishments must disclose on the product label the percentage of the retained moisture (USDA-FSIS, 2001).

Chilling carcasses should be rapid enough to restrict microbial growth or injure microbial cells. In the United States, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has recommended that deep muscle tissue (15 cm) of food animal carcasses should decrease to at least 7.2°C within 36 h, with a temperature of at least 10°C reached within the first 24 h of chilling (NACMCF, 1993). Typically, United States beef slaughter facilities use a two-stage chilling process. First, the carcasses are held in a cooler for 12 h at approximately –3°C and sprayed periodically (up to 2 min spraying duration every 30 min) with water or antimicrobial solutions (2°C) (Stopforth et al., 2004). This step, known as spray chilling, induces the rapid chilling of carcasses due to water evaporation from carcass surfaces. The second stage of the chilling process involves the storage of carcasses at about 1°C for the additional 24–38 h prior to fabrication.

The spray chilling process was first designed by Swift & Company in 1975 (Heitter, 1975), and thereafter became widely employed by the United States meat packing industry. Initially, the developed process involved the spray chilling of beef carcasses with 50 ppm of chlorine, as the antimicrobial ingredient (Heitter, 1975). As indicated in a previous section of this chapter, chlorine is not currently permitted for the direct application to red meat carcasses. Typically, modern United States meat packing plants may spray carcasses with lactic or acetic acid solutions during chilling. Hamby et al. (1987) showed that intermittent spraying (30-s spray every hour during the 12-h period) of beef carcasses with 1% of lactic or acetic acid during chilling significantly reduced APC on carcass surfaces. Similarly, Dickson (1991) reported that acetic acid spray chilling of lean and fat beef tissue inoculated with *S*. Typhimurium, *L. monocytogenes*, and *E. coli* O157:H7 reduced the pathogen numbers by up to 3 log-cycles. Stopforth et al. (2004) evaluated several antimicrobial compounds which were approved by the USDA-FSIS for the spraying of beef caresses as potential "in-cooler" intervention during the simulated two-stages chilling process (12 h at –3°C plus intermittent spraying followed by 24–38 h at 1°C). It was reported that residual levels of the chemicals, which were sprayed on carcasses during the initial stage of chilling, were continuously reducing numbers of *E. coli* O157:H7 on inoculated beef carcass tissue (Stopforth et al., 2004). Among evaluated compounds (CPC, ammonium hydroxide, lactic acid, ASC, peroxyacetic acid, sodium hydroxide, and sodium hypochlorite), 0.1 or 0.5% CPC treatment was the most effective, reducing pathogen numbers by more than 5 log-cycles after 24 h of chilling. These results exemplified the multiple hurdle decontamination approach since the injury/death of the pathogenic cells caused by the antimicrobials was magnified by the injury which was caused by the evaporative cooling (Stopforth et al., 2004).

Carcass Spraying Before Fabrication

In recent years, USDA-FSIS (2008) has approved and certain major United States meat processors spray chilled carcasses with solutions of organic acids (e.g., lactic), ACS, or peroxyacetic acid-based preparations immediately before cutting into primals and packaging. This decontamination step is applied as an extra antimicrobial hurdle to improve the microbiological quality of the product and to control levels of contamination accumulating during the fabrication process (King et al., 2005; Gill & Badoni, 2004). However, Bacon, Sofos, Belk, and Smith (2001) showed that post-chill spraying (29.5°C, 3 s) of carcasses with low concentrations of lactic acid (1.5–2.5%) failed to reduce microbial contamination, presumably because bacterial cells become irreversibly attached to carcass surfaces, and therefore a more powerful intervention was needed to decrease microbial population. As the efficacy of organic acids increases with temperature, their application may have a stronger antimicrobial effect on hot

beef carcasses compared to post-chilled application because the temperature of the solution decreases as it contacts a chilled carcass.

Multiple Hurdle Approach

On-line decontamination practices used during conversion of food animals to meat evolved over the years as a result of extensive scientific research. This evolution was driven by the need to meet consumer demands for safe products and to comply with federal requirements. As a result, single interventions or antimicrobials used to improve microbial quality are currently applied in a logical manner, sequentially or in combinations. Such an approach is referred to as a multiple barrier or hurdle technology and delivers a combined or synergistic decontamination effect (Leistner & Gorris, 1995). In addition, the use of the hurdle technology allows for the use of single interventions or antimicrobials at a lower intensity, decreasing or negating adverse effects on organoleptical quality attributes.

The multiple hurdle pathogen control approach is also widely applied in carcass decontamination in the United States. Graves Delmore, Sofos, Schmidt, and Smith (1998) demonstrated that sequential application of pre-evisceration washing, acetic acid rinsing, final washing, and final acetic acid rinsing was more effective in reducing microbial contamination on beef adipose tissue, compared to single decontamination treatments. Bacon et al. (2000) evaluated the effect of sequential decontamination interventions applied at eight different beef-slaughtering plants. In that study, pre- and post-evisceration interventions reduced levels *E. coli* by approximately 2 logs, and then chilling decreased microbial levels by another log-cycle (Bacon et al., 2000). Calicioglu, Kaspar, Buege, and Luchansky (2002) demonstrated the efficacy of the hurdle approach using a sequential application of GRAS chemicals for improving the safety of beef carcass quarters and subprimal cuts. The researchers reported that prespraying samples with a 5% solution of Tween 20, a food grade surfactant, enhances reductions of *E. coli* O157:H7 levels caused by 2% lactic acid (Calicioglu et al., 2002). This enhanced reduction is not surprising, as surfactants decrease the surface tension of solutions thereby improving the "wettability" of surfaces allowing for more uniform distribution of the antimicrobial substances and possibly bringing them closer to the bacterial cell surface (Neu, 1996). In addition, the authors suggested that Tween 20 might loosen attachment of bacterial cells to the meat surfaces, therefore making cells more easily removed, injured, or inactivated by the lactic acid treatment (Calicioglu et al., 2002).

In hurdle decontamination technologies, chemical or physical antimicrobials treatments are applied in sequence or simultaneously, inflicting concurrent and variable injuries to bacterial cells. Sequential application of decontamination hurdles involves use of interventions on animal hides, followed by knife trimming, steam vacuuming, pre-evisceration washing, washing, thermal decontamination

with water or steam, organic acid rinsing, chilling, and chemical spraying before deboning. The simultaneous hurdle approach in carcass decontamination includes use of warm acid solutions (heat and acid) and steam vacuuming (heat and vacuum, water, physical removal) (Sofos, 2005; Sofos & Smith, 1998; Stopforth & Sofos, 2005).

Future Trends

A need for improvements in food safety and consumer protection, regulatory requirements and standards, trade specifications, and recommendations for processors have induced intensified research in the meat safety area. The constantly increasing amount and depth of the available scientific information and subsequent implementation of new processes, antimicrobials, and optimization of existing ones have led to substantial improvements in the microbial quality of meats. However, the complete elimination of pathogens of public health concern may not be achievable in raw meat. Microbial quality of meat and poultry products will always be a challenge to processors due to the need for control of traditional as well as "new," "emerging," or "evolving" pathogens, which may have increased virulence or acquire resistance to antimicrobials or stresses caused by processing interventions (Sofos, 2008).

Future research trends should include development and optimization of the following areas: molecular investigative methods for pathogen monitoring throughout the processing continuum; methods for in-plant evaluation of efficacy of antimicrobial compounds and processes; susceptibility of multidrug-resistant pathogens to antimicrobial interventions; pathogen stress adaptation, cross-protection, and identification of conditions that contribute to, or control this; and determination of the prevalence of new pathogens throughout the food chain and evaluation of their potential impact on human health. The appropriate use of currently available scientific information and of new knowledge gained from future research is expected to contribute to better risk assessments and regulatory decision making as well as to advances in on-line meat decontamination, pathogen control and food safety. This will occur by achieving continued decreases in pathogen prevalence and levels on fabricated carcasses and meat products.

Currently, 20–28% of small and very small meat packing plants in the United States do not use any carcass decontamination interventions (Cates et al., 2008). It is expected that, in the future, decontamination interventions will be more widely accepted by this segment of the industry. The use of spray washing with water or antimicrobials, steam vacuuming, and steam pasteurization will continue to gain acceptance by the industry. A growing consumer demand for organic, natural, and minimally processed meat and poultry products will continue to induce the identification of new and re-evaluation of

existing natural antimicrobials and their use. Other trends will include modernization and redesign of existing slaughter plants, construction of new facilities with improved air flow and product/personnel traffic control. Processing methods will continue to evolve and become more automated, energy efficient, resulting in decreased levels of carcass handling by plant employees, and in products of more consistent microbial quality.

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Chapter 7
Advanced Decontamination Technologies: High Hydrostatic Pressure on Meat Products

Margarita Garriga and Teresa Aymerich

Introduction

The increasing demand for "natural" foodstuffs, free from chemical additives, and preservatives has triggered novel approaches in food technology developments. In the last decade, practical use of high-pressure processing (HPP) made this emerging non-thermal technology very attractive from a commercial point of view. Despite the fact that the investment is still high, the resulting value-added products, with an extended and safe shelf-life, will fulfil the wishes of consumers who prefer preservative-free minimally processed foods, retaining sensorial characteristics of freshness. Moreover, unlike thermal treatment, pressure treatment is not time/mass dependant, thus reducing the time of processing.

HPP of foods was first reported by Hite (1899). After a treatment of milk at 670 MPa for 10 min a 5–6 log-cycle reduction in total counts was achieved, and meat treated at 530 MPa for 1 h showed insignificant microbial growth after 3 weeks. In 1914, Bridgman reported egg albumen coagulation at 590 MPa for 1 h. These pioneers' observations suggested that high hydrostatic pressure (HHP) was a useful tool for food preservation, however, the technology was ignored until the 1970 s when it was applied to improve the tenderness of meat by Australian meat scientists (Macfarlane, 1973). Later the technology attracted a lot of interest and large HPP research programs were established in Japan, Europe, and United States. Its capacity to preserve the essential, functional, and nutritional characteristics of the food products while ensuring food safety, and the fact that the changes induced in the food proceed in a different manner from the properties of food processing by heat, were important factors in the re-emergence of the HPP food technology (Mozhaev, Heremans, Frank, Masson, & Balny, 1996; Cheftel & Culioli, 1997). The effect of pressure on several meat characteristics of meat and meat products has been

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published (Cheftel & Culioli, 1997; Ledward, 1998; Suzuki, Kim, Tanji, Nishiumi, & Ikeuchi, 2006). Although HHP cannot be universally applied to all types of food, this technology has enormous potential both alone or combined with other technologies (gamma irradiation, alternating current, ultrasound, and carbon dioxide or antimicrobial treatment), also for the development of novel foods. Key challenges should be addressed and identified (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007).

Some companies, mainly in the United States and Japan, are currently commercializing pressurized products (Table 7.1). Regarding meat products, cooked ham, cured ham, some precooked meals with turkey and delicatessen (*sausage tapas*), chicken and pork cuts, precooked meals with poultry, cooked and cured ham, Parma ham, mortadella, bacon, salami, and other smoked or non-smoked sausages are available in the market (Fig. 7.1). Generally a treatment of 600 MPa during 2–10 min is considered. Despite the high initial investment, the processing cost has been estimated at 14 eurocent per kg of product treated at 600 MPa, including investment and operating costs (Fig. 7.2). At a commercial plant with a 300 L pilot capacity, 4.51 cycles per

Table 7.1 Pressurized food products commercially available

Products	Company name	Location
Ready-to-eat Meat Products	Campofrío Alimentación, S.A.	Spain
”	Esteban Espuña, S.A.	Spain
”	Vismara (Ferrarini)	Italy
”	Abraham	Germany
”	Fuji Mutterham	Japan
”	Hormel Foods Corp.	USA
Ready-to-eat Chicken	Perdue Farms, Inc	USA
”	Juárez Foods	USA
Jams, fruit toppings or blends	Meidi-ya	Japan
”	Tanisyo Ltd	Japan
”	Nisshin Foods	Japan
”	Leahy Orchards Inc	Canada
”	Ata	Italy
Fruit smoothies or juices	Avomex Inc	USA
”	Lovitt Farms Inc	USA
”	Ata, Ortogel	Italy
”	Pokka, Chyonosono	Japan
”	Frubaça	Portugal
”	Pampryl	France
Guacamole	Calavo Growers, Inc	USA
”	Avomex, Inc	USA
Hummus, Dips, Spreads	Hannah International	USA
Oysters	Joey Oysters, Inc	USA
”	Motivatit Seafoods, Inc	USA
”	Oysa	Australia
”	Kivun	Japan

Fig. 7.1 HPP-treated meat product manufactured by a Spanish meat company

Fig. 7.2 NC Hyperbaric industrial equipment for HPP up to 650 MPa located at IRTA-CENTA (Monells). About 120 L capacity, 300 mm inner diameter

hour could be carried out. Products treated by HHP do not need specific label in United States. The technology is well accepted in Europe as an alternative technology, despite the general lack of consumer awareness of "high-pressure processing" (HPP). Baron et al. (1999) reported 67% acceptability by consumers from three different European countries (France, Germany and United Kingdom). In more recent studies, the results of a conjoint analysis to assess the importance of various product and marketing factors on consumer interest in foods processed by emerging technologies showed that HPP has a strong positive influence on consumer interest, compared with irradiation and genetic modification (Cardello, Schutz, & Lesher, 2007).

General Effects of HHP

Food enzymes can undergo reversible or irreversible pressure-induced changes resulting in partial or complete activation or inactivation (Cheftel, 1995). The denaturation of proteins by pressure seems to allow the destabilization of non-covalent interactions in the tertiary structure (Pittia, Wilde, Husband, & Clark, 1996; Tedford, Kelly, Price, & Schaschke, 1999) and although these proteins structurally retain much of their secondary structure, the small degree of unfolding that exposes the hydrophobic regions of the protein could be the cause of protein aggregation (Mozhaev et al., 1996; Tedford et al., 1999). But the nutritional value, vitamins, and the majority of small substances responsible for the flavors of the products are retained. This is viewed as an important benefit for the food industry (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989; Smelt, 1998; Téllez, Ramírez, Pérez, Vázquez, & Simal, 2001) and in

general minimal modifications in the sensory characteristics of the product are introduced, especially in cooked and cured meat products. Nevertheless, it has been reported that the pressurization of post-mortem beef meat could modify the enzymatic system (Homma, Ikeuchi, & Suzuki, 1994; Jung, De Lamballerie-Anton, & Taylor, 2000), the texture and ultrastructure (Macfarlane, 1985; Suzuki, Kim, Homma, Ikeuchi, & Saito, 1992), the gelation properties of myofibrillar proteins (Ikeuchi, Tanji, Kim, & Suzuki, 1992), and the microbiological quality of meat (Cheftel & Culioli, 1997). During the last decade, to overcome these drawbacks, different applications of high-pressure–low-temperature combinations were investigated, including high-pressure freezing or thawing and storage at subzero temperatures under pressure (Otero, 1999; Sanz, 2005). Low-temperature pressurization of frozen meat was patented as a system to prevent color degradation (Arnau et al., 2006).

Effect of HHP on Microorganisms

High pressure induces several changes in the cell membrane and cell wall of microorganisms, including separation of the cell membrane from the cell wall, contraction of the cell membrane, compression of gas vacuoles, cell lengthening, and release of intracellular material (Patterson, 2005). Ribosome dissociation was also shown to limit cell viability at high pressures (Abe, 2007).

Moderate levels of pressure decrease the rate of growth and reproduction, whereas very high pressures cause inactivation, the threshold depending on the microorganism and species. Yeasts and moulds are relatively pressure sensitive; however, ascospores of heat-resistant moulds such as *Byssochlamys*, *Neosartorya*, and *Talaromyces* are generally considered to be extremely HHP resistant (Chapman, Winley, & Fong, 2007; Smelt, 1998). In general, Gram-negative bacteria and cells in lag phase are more sensitive than Gram-positive and stationary phase cells. Vegetative pathogens like *Vibrio* and *Yersinia* are relatively sensitive to pressure and can be inactivated at pressures less than 350 MPa, whereas *Staphylococcus aureus* needs pressures higher than 500 MPa (Chen, Guan, & Hoover, 2006). Nevertheless and according to Schreck, Layh-Schmidt, and Ludwig (1999) barotolerance could not be correlated with the Gram type and the presence of cell wall. Most pressure sensitive bacteria are rod or spiral shaped, whereas the most resistant ones are spheres. Medium sensitive bacteria exhibit a mixed assortment of forms between short rods and cocci (pleomorphic shape). Ludwig, van Almsick, and Schreck (2002) even concluded that the presence of a cell wall might be disadvantageous for a bacteria species when exposed to high pressure. More recently, Hartmann, Mathmann, and Delgado (2006) confirmed that pressure load on the cell wall induces severe non-hydrostatic stress which might interact with inactivation mechanisms such as denaturation of membrane-bound proteins.

Endospores, when compared with vegetative cells, tend to be extremely HHP resistant, *Clostridium* endospores being more pressure resistant than *Bacillus*. However, bacterial spores can be inactivated by first inducing spore germination using relatively low pressure, followed by complete inactivation and death of the spores using relatively mild heat treatments (Smelt, 1998) or subsequent pressure treatments (Wuytack, Boven, & Michiels, 1998). Different combinations of temperature, time, pressure, and cycling treatments were studied and it was reported that the complete efficacy for achieving spore inactivation depends on several factors (Farkas & Hoover, 2000; Torres & Velazquez, 2005).

Generally, the prions associated to neurological disorders are even more difficult to destroy than bacterial spores. Some prions are affected by pressure combined with a simultaneous heat treatment at 60°C (García et al., 2004). Pressure resistance of viruses varies considerably; HHP can cause damage to the virus envelope preventing the virus particles from binding to cells or even complete dissociation of virus particles, which may be either fully reversible or irreversible (Hogan, Kelly, & Sun, 2005).

Other factors influencing threshold of inactivation are the pressure applied, the time of processing, the composition of the food, temperature, pH and water activity (Tewari, Jayas, & Holley, 1999). In addition, pressure resistance of microorganisms would be reinforced in rich nutrient media (Hoover et al., 1989). Carbohydrates, proteins, and lipids have a protective effect (Simpson & Gilmour, 1997). This indicates that validation processes in real products are required. Because the costs of high-pressure processing and throughput are related to treatment pressure, time, and temperature, further studies are needed to help food processors to select optimum processing conditions to be commercially viable. Cell death increases as pressure level increases but not following a first-order kinetics, as a tail of inactivation is sometimes recorded (Garriga, Aymerich, Costa, Monfort, & Hugas, 2002; Kalchayanand, Sikes, Dunne, & Ray, 1998b). Sublethally injured cells recovered during storage and grew (Aymerich, Jofré, Garriga, & Hugas, 2005; Chen & Hoover, 2003; Garriga et al., 2002; Patterson, Quinn, Simpson, & Gilmour, 1995). Depending on the food product, the preservation conditions, and duration, the microbial proliferation could reach very high proportions and could also cause the formation of high levels of biogenic amines (Ruiz-Capillas & Jiménez-Colmenero, 2004). Some biogenic amines (tyramine and histamine, directly, or putrescine and cadaverine, indirectly) can pose health risks due to toxicological effects, when ingested in large quantities. Furthermore, there is some evidence that these technologies can condition the amine profile produced. Therefore, more in-depth studies during the shelf-life of the products are necessary to determine how the different factors associated with these technologies can affect the formation of these compounds and the recovery of microbial cells.

High Hydrostatic Treatments to Improve the Food Safety of Different Types of Meat Products

Raw Meats

In order to extend the shelf-life, increase the food safety and quality of raw meat, alternative non-thermal technologies such as high hydrostatic pressure alone or combined with active packaging and natural biopreservatives have been studied.

HHP was reported as being able to reduce 6–7 log CFU/g the total counts in meat homogeneate and more than 4 log CFU/g in minced beef muscle and mechanically recovered poultry meat, when a ca. 400 MPa treatment was assayed (Carlez, Rosec, Richard, & Cheftel, 1994; Shigehisa, Ohmori, Saito, Taji, & Hayashi, 1991; Yuste et al., 2001). When a higher treatment (500 MPa) was applied in poultry sausages, a total count reduction equal to the pasteurization process was obtained (Yuste, Pla, & Mor-Mur, 2000). *Toxoplasma gondii* cysts were inactivated in a ground pork meat with an HHP of 300 MPa (Lindsay, Collins, Holliman, Flick, & Dubey, 2006). A 700 MPa treatment was able to reduce 5 log CFU/g the counts of *E. coli* O157:H7 in raw minced meat (Gola, Mutti, Manganelli, Squarcina, & Rovere, 2000).

Marinated beef loin is a raw meat product with high a_w (0.98), low level of salt (1%), and a mixed microflora with an important initial contamination, around 6 log CFU/g (Garriga, Grèbol, Aymerich, Monfort, & Hugas, 2004). HHP treatment of sliced marinated beef loin at 600 MPa for 6 min was very effective in reducing all the microbial groups investigated, achieving a reduction of 4 log cycles after treatment for aerobic, psycrotrophic, and LAB counts and nearly 3 log CFU/g for *Enterobacteriaceae*. No further recovery of survivors were recorded during 120-day storage at 4°C, while untreated samples reached 8 log CFU/g after 30 days of storage. Regarding pathogens, 9 out of 15 control samples (untreated) showed presence in 25 g of *L. monocytogenes* and *Salmonella* spp. Moreover all of the HPP samples ($n = 15$) recorded absence of either *L. monocytogenes* or *Salmonella* in 25 g during the whole 4°C storage period (120 days) (Garriga et al., 2004). From a safety point of view these results confirm that HPP is a powerful tool to control risks associated with these pathogens in raw meats. In fact challenge tests performed in our laboratory showed that pressurization at 600 MPa for 6 min was capable of reducing from ca. 3.5 log CFU/g the initial spiked counts of *Salmonella*, *L. monocytogenes*, *C. jejuni*, *Y. enterocolitica* to values below the detection limit during the whole 4°C storage (120 days) of treated marinated beef loin slices.

Morales, Calzada, and Avila (2008) investigated the effect of single- and multiple-cycle HHP treatments at 400 MPa on the inactivation of *E. coli* O157:H7 inoculated (ca. 7 log CFU/g) into ground beef. The authors concluded that multiple-cycle HHP treatments achieved a higher lethality than did single-cycle treatments for the same total length of treatment (including come up and

depressurization times) or the same lethality for a shorter total length of treatment. Changes in the color and texture of ground beef caused by single- and multiple-cycle HP treatments of the same lethality (i.e., one 20-min cycle and four 1-min cycles) were similar. Luscher, Balasa, Frohling, Ananta, and Knorr (2004) and Luscher, Sunderhoff, Urrutia Benet, and Knorr (2005) reported a 2–3 log-cycle bacterial reduction in frozen minced beef and in frozen suspensions, respectively, due to the mechanical stress associated to phase transitions (ice I to ice II or III).

Several authors observed undesirable color modifications in pressurized samples: in marinated beef loin at 600 MPa (Garriga et al., 2004), in minced beef muscle treated at pressures higher than 350 MPa (Carlez, Veciana-Nogués, & Cheftel, 1995), and in shear forces and hardness of *semitendinosus* (ST) muscle between 100 and 500 MPa (Lee, Kim, & Lee, 2007). Nevertheless, no differences in consumers' acceptance of ready-to-eat low-fat pastrani, Strassburg and Cajun beef were reported (Hayman, Baxter, O'Riordan, & Stewart, 2004). Contractile myofibrillar proteins are thought to be primarily responsible for differences in the textural properties of HHP-treated meat. An increase in the hardness of beef muscle treated at 20°C with pressure levels up to 400 MPa and a slight decrease at higher pressures were reported (Ma & Ledward, 2004), ultrastructural changes in myofibrils becoming visible at pressures above 325 MPa (Jung, De Lamballerie, & Ghoul, 2000). On the other hand, HHP treatment affects the integrity of lysosomes (D. S. Jung et al., 2000) and increases cathepsin D and acid phosphatase activities in pressurized beef, influencing its textural characteristics (D. S. Jung et al., 2000).

Cured Meat Products

The microflora of dry-cured ham due to their low water activity (0.89) and high salt content (4.6%) are mainly composed of GCC+ and yeasts, which are also present in the product after slicing. Garriga et al. (2004) reported a 2 log cycle decrease of total bacteria counts after an HHP treatment at 600 MPa for 6 min of vacuum-skin-packaged dry-cured ham slices. The counts maintained around 3 log CFU/g till the end of storage (120 days at 4°C). Psychrotrophs showed higher pressure sensitivity compared to mesophiles not recovering their ability to grow during storage. Regarding yeasts, although no growth was observed in non-treated samples during storage, the counts of HHP-treated samples maintained the levels achieved after treatment (<1 log CFU/g) during the whole period studied. *Salmonella* and *Campylobacter* were not detected in any samples neither control nor HHP treated, whereas *L. monocytogenes* were present in one of the control samples but absent in all HHP-treated samples during the whole storage period studied (Garriga et al., 2004). In a challenge test, when *Salmonella* and *L. monocytogenes* were spiked between dry-cured ham slices both pathogens were highly inactivated after pressure treatment (600 MPa 6 min)

and the counts were reduced from ca. 3.5 log CFU/g to <10 CFU/g (Jofré, Aymerich, Monfort, & Garriga, 2008). In a previous work at the same pressure/time different inactivation levels after HHP treatment were observed in dry-cured ham spiked with *L. monocytogenes*, depending on the equipment used (Hugas, Garriga, & Monfort, 2002).

Treatment of sliced Iberian and Serrano hams at 450 MPa for 10 min significantly reduced the population of *L. monocytogenes* Scott A, spiked at ca. 6 log CFU/g. After 60 days at 4°C or 8°C, the counts were 3.24 and 4.70 log CFU/g in HPP and control, respectively, for Iberian and 2.73 and 5.07 log CFU/g for Serrano ham (Morales, Calzada, & Nuñez, 2006). The color parameters L^* and a^* were not influenced by high-pressure treatment, and parameter b^* increased only in Iberian ham. By contrast, a few studies have reported pressure-induced color changes in both lightness (increased) and redness (decreased) when applied to Parma ham (Tanzi et al., 2004) and Iberian dry-cured ham (Andrés, Adamsen, Moller, Ruiz, & Skibsted, 2006). Moreover, Tanzi et al. (2004) reported some texture and flavour changes in the pressurized samples. An enhanced perception of saltiness was reported by Saccani, Parolari, Tanzi, and Rabbuti (2004) after a treatment of 600 MPa for 9 min.

Serra, Grèbol, et al. (2007) described the effect of HPP (400 and 600 MPa) applied to frozen hams at different stages of the drying process. HHP-treated hams showed slightly lower visual color intensity than the control ones. In general, pressurization did not have a significant effect on the flavour characteristics of the final product as reported by other authors (Morales et al., 2006). The 600 MPa-hams from the ERS process (at the end of the resting stage) showed significantly lower crumbliness and higher fibrousness scores than the control and the 400 MPa, without negatively affecting the overall sensory quality of the hams. An increase in lightness L^* was only observed in the *biceps femoris* muscle from green hams (at the early stages) at both pressures studied. Generally, only a little or no decrease in redness has been reported after pressurization, because of the protective action of nitric oxide on myoglobin, i.e., the nitrosylmyoglobin formation protects the pigment against oxidation, thus preserving the cured color (Carlez et al., 1995; Cheftel & Culioli, 1997; Farkas et al., 2002). Serra, Sárraga, et al. (2007) demonstrated that high-pressure treatment (400 and 600 MPa) slightly reduced antioxidant enzyme activity in dry-cured hams.

Another traditional Spanish product, manufactured similarly to dry-cured ham is *Cecina de León*, an intermediate moisture beef meat product with the typical red color, smoked flavor and slightly salty taste. At the end of drying the microbial counts are in general low, around 3 log CFU/g, but after slicing and packaging operations cross-contamination leads to an increase of the total counts which reduces the expected shelf-life of this product. Rubio, Martínez, García-Gachán, Rovira, and Jaime (2007a) studied the application of a 500 MPa pressure treatment for 5 min in order to extend the shelf-life of *Cecina de León*. A delay of the growth of spoilage flora was achieved with a subsequent extension of the shelf-life to 210 days, compared to the 90 days usually expected.

However, the treatment did not avoid sensory changes during storage, limiting the optimum storage time to 90 days. This result agrees with those of Andrés, Møller, Adamsen, and Skibsted (2004) which were unable to detect any differences in TBA values between untreated and HPP (400 MPa and 15 min) Iberian ham. However, Cava, Tárrega, Ramírez, Mingoarranz, and Carrasco (2005) pointed out TBA values on Iberian ham treated with high pressure (200–300 MPa and 15–30 min) increased, although after storage for 90 days, similar TBA values were found on treated and untreated samples. On the contrary, Saccani et al. (2004) reported that the HPP (600 MPa during 3, 6, or 9 min) modified the sensory parameters (loss of color intensity, saltier taste and greater firmness) of dry-cured hams that had undergone 14 and 18 months of ripening.

Cooked Meat Products

Due to its composition, pH, water activity, and lack of endogenous microflora, sliced cooked meat products may not represent a major hurdle for microbiological growth during refrigerated storage if recontamination during slicing and packaging occurs. Its shelf-life depends on good manufacturing practices, the use of white rooms, and the post-pasteurization process. In this sense, HPP may represent an efficient alternative post-processing technique to increase the shelf-life of these products without significant sensory modifications.

The effect of physico-chemical and sensorial changes were mainly studied in cooked ham. No color and no pH changes have been reported in cooked ham treated by HHP (Carpi et al., 1999; Cheftel & Culioli, 1997; Hayman et al., 2004; López-Caballero, Carballo, & Jiménez-Colmenero, 1999). Even when cooked ham was stored for 8 weeks at 4–6°C after a 300–600 MPa/10–30 min/room temperature treatment, no changes in texture or color of cooked ham were reported throughout storage (Karlowski, Windyga, & Fonberg-Broczek, 2002). Moreover, Hugas et al. (2002) reported that the overall physico-chemical composition of cooked ham was not significantly affected after a treatment of 600 MPa for 10 min at 30°C. The non-proteic nitrogen fraction and aminoacid content were equivalent, fatty acid composition, and cholesterol content were kept and contents of vitamins from group B were not modified. Mineral composition was similar and only a decrease of the calcium content was observed. No changes in bioavailability of nutrients and no increase in the solubility of cytoplasmatic proteins were observed.

In vacuum-packaged-cooked sausages, Mor-Mur and Yuste (2003) also reported that color attributes did not change when the product was treated at 500 MPa for 5 or 15 min at mild temperature (65°C). When color, texture, and yield of pressure-treated sausages were compared to sausages treated with a conventional heat pasteurisation (80–85°C for 40 min), pressurised sausages were more cohesive and less firm than heat-treated sausages. HHP induced

higher yield than heat treatment. Sensory analysis did not detect differences between both types of sausages; and even when there were differences, pressurized samples were preferred in more occasions because of their better appearance, taste and, especially, texture. The effects of an HPP at 600 MPa, 10 min, 20°C on the quality of cooked pork ham prepared with two different levels of curing ingredients in brine and stored in refrigeration (4–6°C) for 8 weeks have been also evaluated by Pietrzak, Fonberg-Broczek, and Mucka (2007). HPP causes significant improvement of shelf-life of vacuum-packed ham, including the samples with reduced level of curing ingredients in brine to 8 weeks in refrigerator conditions. HPP did not affect the texture or color of ham, but it increased the drip loss during storage in the packed samples. This may indicate that HPP has negative effects on water holding capacity of cooked products.

Concerning microbiological food safety, different assays have been performed in meat models and different food matrices. In a meat model system, Garriga et al. (2002) reported the application of HHP treatment of 400 MPa for 10 min at 17°C. *E. coli* displayed a 4–5 log cycle decline after 24 h of pressurization but it recovered and grew to 10^6–10^7 CFU/g at the end of storage at 4°C. A 6 log reduction after treatment was observed for *Salmonella*, *L. monocytogenes*, slime producing LAB (*Lactobacillus sakei* and *Leuconostoc carnosum*) but while *Salmonella* was not able to recover during refrigerated storage, the other challenged bacteria quickly recovered after treatment, reaching initial inoculated counts. *S. aureus* was the species least sensitive to the HPP treatment.

In sliced-cooked ham several assays have been performed by different authors to assess the effectiveness of different high hydrostatic treatments at different temperatures of treatment and shelf-storage and interleaver application to avoid release of meat juices and fat, on naturally contaminated and artificially spiked spoilage and pathogenic microorganisms. López-Caballero et al. (1999) studied the efficiency of a treatment of 200–400 MPa for 5 and 20 min at 7°C in prepackaged naturally contaminated sliced-cooked ham when stored at 2°C for 35 days during post-processing, slicing, and packaging. The treatment at 400 MPa for 20 min was able to reduce total viable counts in 2 log CFU/g, keeping these levels until the end of the storage. LAB were not detected until day 21 and GCC+ were under the detection limit at day 35. The 400 MPa 5 and 20 min treatments were also better than the 200 MPa 20 min treatment to extend the detection of *Enterobacteriaceae* and *Brochothrix thermosphacta*, respectively, until the day 35 when compared to the 7th and 21st days of detection of the 200 MPa treatment. In a second trial, the same authors (López-Caballero, Carballo, Solas, & Jiménez-Colmenero, 2002) assayed the effect of combined treatments of high pressure (300 MPa, for 15 min) and temperature (5, 20, 35, and 50°C) on microbial inactivation (total bacterial count, LAB, Baird Parker microflora, *Pseudomonas* sp., and *Enterobacteriaceae*) and color, in naturally contaminated sliced cooked ham. Greater pressure-induced loss viability was observed in Gram-negative bacteria. Microbial inactivation was more pronounced when pressurization was applied at 50°C. Microbial sensitivity to the HPP was conditioned by the processing temperature, the

microorganism group, and the type of meat product. The effectiveness of a higher pressure treatment, 600 MPa 6 min at 31°C, to avoid growth of endogenous, non-inoculated yeasts and *Enterobacteriaceae*, for delaying the growth of LAB and to reduce the risks associated to *Salmonella* and *L. monocytogenes* in sliced cooked ham were also reported by Garriga et al. (2004). Later, Aymerich et al. (2005) reported the effect of a treatment of 400 MPa 10 min 17°C on *Salmonella* and *L. monocytogenes* artificially spiked in vacuum-packaged sliced cooked ham. The treatment was able to diminish the spiked cells (3×10^2 CFU/g) under 4 MPN/g for *Salmonella* and the growth of *L. monocytogenes* inhibited until 40 days of refrigerated storage at 1 or 6°C. After that period, and at 6°C, *L. monocytogenes* was able to grow until counts similar to that of non-pressurized samples (ca. 8 log CFU/g), while at 1°C kept to the low levels achieved after pressurization. The effect of the presence of an interleaver to avoid release of meat juices and fat in spiked sliced cooked ham with *L. monocytogenes* and *Salmonella* at 3×10^4 CFU/g was also studied by Jofré, Garriga, and Aymerich (2007) and Jofré, Aymerich, and Garriga (2008). A three-layer interleaver was placed between the slices, vacuum packaged, and HHP treated at 400 MPa. While in non-pressurized samples, *L. monocytogenes* grew until ca. 10^8 CFU/g under refrigerated storage at 6°C, in pressurised samples at 400 MPa 10 min 17°C, an initial decontamination of the pathogen of 1.76 log CFU/g was observed and counts progressively increased after day 30 to levels of 6.5 log CFU/g. *Salmonella* diminished under 10 CFU/g, a value that was maintained for 3 months of storage at 6 C. The efficiency of an HPP (400 MPa for 10 min 17°C) in sliced cooked ham was also evaluated after a cold chain break when combined with different refrigeration temperatures (Marcos, Jofré, Aymerich, Monfort, & Garriga, 2008). The treatment caused an immediate reduction of *L. monocytogenes* counts in a range of ca. 3 log CFU/g but regrowth, specially at 6°C, was recorded. Levels after the cold chain break reached the same high levels (8 log CFU/g) as without pressurization. At 1°C, a slight regrowth was observed after pressurization but, even with a cold chain break, the counts did not exceed the initial counts and the treatment achieved ca. 2 log CFU/g lower counts than without pressurization. The presence of high-stressed cells that were not able to achieve the same growth rate as at 6°C may be the cause. Afterwards, the effectiveness of higher pressure treatments at 600 MPa was evaluated in sliced cooked ham spiked with 4 log CFU/g of *Salmonella* sp., *L. monocytogenes*, and *S. aureus*, after 3 months of storage at 1° and 6°C (Jofré, Garriga, & Aymerich, 2008). The application of pressure reduced the levels of *Salmonella* and *L. monocytogenes* to levels below 10 CFU/g. These levels continued until the end of storage at both 1 and 6°C. HPP reduced the counts *S. aureus* by less than 1 log cycle.

Some other products such as pork marengo, *Morcilla de Burgos*, and Frankfurters have been considered for HPP. The improvement of microbial quality of pork marengo (a low acidic particulate meat product) after a high-pressure treatment of 400 MPa for 30 min at 20° or 50°C was evaluated by Moerman (2005). Several *Clostridium* spp. and *Bacillus* spp. survived the treatment, and

the Gram-positive cocci *Enterococcus faecalis* and *S. aureus* were revealed to be more pressure resistant than *Saccharomyces cerevisiae* and the Gram-negative bacteria *Pseudomonas fluorescens* and *E. coli*. In commercially sterile sausage, Chung, Vurma, Turek, Chism, and Yousef (2005) reported the effect of HPP (600 MPa, 28°C, 5 min) against barotolerant *L. monocytogenes* inoculated at 10^6–10^7 CFU/g. Inactivation was evaluated after sample enrichment to detect the viability of the pathogen after the treatments. HPP caused a modest decrease in the number of positive samples.

In *Morcilla de Burgos*, the most traditional and famous blood sausage in Spain, Díez, Santos, Jaime, and Rovira (2008) studied the effect of HPP of 300–600 MPa 15°C 10 min, during the chilled storage (28 days). A decrease of *Enterobacteriaceae* and *Pseudomonas* spp. counts below the detection level, $<10^2$ and <10 CFU/g, respectively, was achieved for all the pressures applied, but a treatment of 600 MPa was necessary to reduce the LAB counts in 1 log CFU/g. These microbiological changes seemed sufficient to reduce the sour taste, presence of slime, and vacuum loss until day 15 and to improve the shelf-life of *morcilla de Burgos* to 28 days of vacuum-packed storage at 4°C, in comparison with control samples, possibly due to the injury provoked by the treatment and the storage conditions. LAB recovered after day 7 and reached values of 10^8 CFU/g at day 35. LAB species were differentially affected by HPP at 600 MPa (Díez, Urso, & Rantsiou, 2008) as shown by DNA and RNA-DGGE (PCR-denaturing gradient gel electrophoresis) microbial dynamics analysis, *Leuconostoc mesenteroides* was completely inactivated by the HPP treatment while *Weissella viridescens* was able to recover and carry out the typical spoilage of the product.

In vacuum-packaged Frankfurters, Ruiz-Capillas, Jiménez-Colmenero, Carrascosa, and Muñoz (2007) reported the effect of HPP on the formation of polyamines, microorganism inactivation, and physico-chemical characteristics on the product. The consequences of these treatments were also evaluated throughout chilled storage (2°C) for up to 141 days. Pressurization (400 MPa 10 min 30°C) caused decreases in the levels of total viable and LAB counts by ca. 2 log CFU/g and kept stable and no growth was observed until the end of the 141 days of chilled storage. *Enterobacteriaceae* were kept below the detection limit (<1 log CFU/g). A significant decrease was observed in hardness and chewiness throughout storage. No changes in polyamines were observed.

From the results obtained in the different research studies presented we could conclude that HPP could be recommended as a suitable treatment after post-processing to improve food safety of cooked meat products, without significantly altering sensorial properties.

Fermented Meat Products

In acidic fermented sausages, the fermentation and acidification process that happens during fermentation as a result of LAB growth, together with the

additives added and the decrease of water activity during ripening, is enough to avoid undesirable microbial growth and transform raw meat into a stable product. Nevertheless, in traditional slightly fermented sausages, the pH barrier is not present and thus some pathogens may grow or simply survive. Moreover, consumption of these traditional meat products marketed after slicing has increased in recent years, contamination of the final product just immediately prior to packaging together with the required longer shelf-life have to be considered. Research is ongoing into new technologies to preserve the products' high nutritional and sensory qualities and their comparability with similar untreated products, while assuring microbiological safety. In that sense, an additional hygienic treatment after processing like HPP seems to have gained potential application in the meat industry to increase safety of these products.

Several authors have tested the efficiency of high hydrostatic processing against pathogenic microorganims and quality markers. Krockel and Muller (2002) reported the effect of HPP (200–800 MPa for 10 min at 0°C) and further storage at 7°C for 44 days in vacuum-packaged sliced Bologna-type sausages, *Gelbwurst* (a "diet bologna" without nitrite) and *Lyoner* (nitrite–containing sausage). After HPP, the bacterial counts were markedly decreased at 400 MPa and above and were below the detection limit at 600 MPa and higher. However, a complete inactivation of all inoculated bacteria (*L. monocytogenes, S. aureus, Serratia marcescens*) was not achieved even at 800 MPa. After enrichment, *S. marcescens* was detected in all samples. At pressures of 400 MPa and higher, the type of sausage-influenced survival, recovery, and subsequent growth of the microorganisms during cold storage. Although bacterial counts directly after treatment were slightly higher for *Lyoner* than for *Gelbwurst*, recovery and growth were much more restricted in *Lyoner*-sausage.

In slightly fermented sausages (*fuet* and *chorizo*) Garriga et al. (2005) reported that when a treatment of 400 MPa 10 min 17°C was applied, the LAB or GCC+ population, neither *Enterococcus* populations were affected, whereas the treatment was able to control the growth of *L. monocytogenes*, to reduce *Enterobacteriaceae*, and kept the biogenic amine content stable. HHP was necessary to ensure absence of artificially spiked *Salmonella*. The low a_w may contribute to the protection of several bacterial groups. The high hydrostatic treatment did not modify the TBARS or color parameters although a slight increase in cohesiveness, springiness, and chewiness was observed (Marcos, Aymerich, Guardia, & Garriga, 2007). When an HPP of 200 MPa was applied to the meat batter after stuffing and just before sausage fermentation, the treatment did not interfere with the ripening performance in terms of acidification, drying, and proteolysis as the inoculated LAB decarboxylase-negative strains were not significantly affected. The treatment also prevented *Enterobacteriaceae* growth and subsequently a strong inhibition of diamine (putrescine and cadaverine) accumulation was observed although not tyramine (Latorre-Moratalla et al., 2007). Nevertheless, when 300 MPa was applied to the meat batter after stuffing, a reduction of the spiked *Salmonella* was observed but a partial inhibition of the endogenous LAB delayed the pH drop and thus

L. monocytogenes growth was favored. Moreover, a discoloration of sausages reflected by an increase in the L^* value (lightness) was observed (Marcos, Aymerich, & Garriga, 2005).

Microbiological, physico-chemical, and sensory properties of three types of sausages, with different composition of fats (control, high oleic, and high linoleic *salchichón*), and their evolution over 210 days of storage under refrigeration after a treatment of 500 MPa for 5 min at 18°C were studied by Rubio, Martínez, García-Cachán, Rovira, and Jaime (2007b). No clear relationship could be established between fatty acid composition and the effectiveness of the treatment. HPP had a slight inhibitory effect on some groups of microorganisms, especially yeasts and moulds, psychrotrophic, and anaerobic bacteria. After treatment, LAB counts showed 1 log-cycle reduction, enterococci only slightly diminished in the high oleic batch while *Micrococcaceae* counts were unaffected. During storage, no clear differences in enterococci, LAB, and *Micrococcaceae* counts were observed between treated and non-treated samples. HPP did not show differences in the physico-chemical and sensory properties of the *salchichón*, even though this product is rich in monounsaturated or polyunsaturated fatty acids, the treatment did not exert an enhancing effect on oxidation during storage. No differences in the color parameters were observed. This is a crucial point, if we consider that color is important in consumer acceptance. Moreover, in Spanish fermented sausage *chorizo*, Ruiz-Capillas, Carballo, and Jiménez-Colmenero (2007) reported that HPP (350 MPa 15 min 20°C) did not affect pH or water activity and reduced by <1 log unit the LAB counts that were kept until 160 days of storage at 2°C. The HPP caused a significant reduction of tyramine, putrescine, and cadaverine levels, while there was a significant increase in spermidine. *Enterobacteriaceae* remained below the detection limit.

While high hydrostatic pressure processing could not be recommended at the initial steps of the production, it could be a technology of choice to improve the food safety of fermented meat products, without significant changes in sensory characteristics when pressure is applied on the ripened product.

Combined Hurdles: Antimicrobials and High Hydrostatic Pressure

The effectiveness of mild preservation technologies is based on the combination of different processes or antimicrobial factors in the so called hurdle technology (Leistner & Gorris, 1995). When microorganisms are confronted with multiple antimicrobial factors the likelihood for survival decreases due to an increase in the energy costs that leads to cell exhaustation and death. In addition, the synergy between different factors may permit a decrease in their dose (Gálvez, Abriouel, López, & Omar, 2007). The use of generally recognized as safe (GRAS) LAB or the antimicrobial compounds they produce (i.e., bacteriocins) is a promising ongoing development in food preservation. In general,

antimicrobials provide an excellent opportunity to incorporate them into a combined preservation system. Synergistic effects with HPP have been reported with antimicrobials, low pH, carbon dioxide, organic acids, vacuum packaging, and chilled storage. The effect of lactate, nisin, enterocins, and sakacin together with chilled storage and HHP treatment on the inactivation of *L. monocytogenes* and other food-borne pathogens was reported (Smid & Gorris, 2007). Different modi of application, addition to the meat batter, surface application, and active packaging have been studied. Also the effectiveness of selected starter cultures and high hydrostatic pressure after ripening was evaluated.

In a meat model system, Garriga et al. (2002) observed the importance of nisin addition to the reduction of the less sensitive genera to HPP, *S. aureus,* and to the inhibition of the regrowth of *E. coli* after HHP treatment. To keep *L. monocytogenes* under 10^2 CFU/g the addition of sakacin K, enterocins, or pediocin (ALTA 2351^{Tm}, Quest International) was needed. An additive effect between HHP treatment (400 MPa 10 min 17°C) and different antimicrobials (lactate, nisin) applied to the meat batter was observed in sliced cooked ham (Aymerich et al., 2005). Nisin and lactate allowed the regrowth of *L. monocytogenes* at 6°C, while lactate exerts a bacteriostatic effect during the whole storage period (three months) at 1°C. The most effective treatment for *L. monocytogenes* was the combination of HPP, lactate, and low-temperature storage. Absence of *Salmonella* in 50% of the samples was only achieved in the batches where HPP and nisin (800 AU/ml) were applied. When two different antimicrobials (enterocins and lactate-diacetate) were tested (Marcos et al., 2008), lactate–diacetate exerted a bacteriostatic effect against *L. monocytogenes* during the whole storage period (three months) at 1 and 6°C, even after temperature abuse. Nevertheless, the combination of low storage temperature (1°C), HPP, and the addition of lactate-diacetate was necessary to reduce the levels of *L. monocytogenes* during storage by 2.7 log CFU/g. The combination of enterocins at 2,400 AU/g, HPP, and 6°C storage temperature was not able to inhibit the growth of *L. monocytogenes* after the cold chain break. On the contrary, at 1°C the combination of HPP with enterocins was more effective than with lactate–diacetate, being able to reduce the population of the pathogen to final counts of 4 MPN/g after 3 months of storage, even after the cold chain break.

The effectiveness of the combination of HPP (600 MPa) with the natural antimicrobials nisin and potassium lactate applied in the meat batter of cooked ham has been evaluated in slices artificially inoculated with 4 log CFU/g of *Salmonella* sp., *L. monocytogenes*, and *S. aureus* after 3-month storage at 1° and 6°C (Jofré, Garriga, et al., 2008). In non-HPP sliced cooked ham, the addition of nisin plus lactate inhibited the growth of *L. monocytogenes* during the entire storage period, while the refrigerated storage inhibited the growth of *Salmonella* sp. and *S. aureus*. The application of HPP was necessary to reduce the levels of *Salmonella* and *L. monocytogenes* to <10 CFU/g, levels that were kept until the end of storage at both 1 and 6°C. HPP only reduced the counts of *S. aureus* less than 1 log cycle and the combination with nisin and refrigeration at 6°C was necessary to decrease the counts of *S. aureus* by 2.4 log CFU/g after 3 months of storage.

Differential efficiency of sprayed surface bacteriocin application combined with an HPP of 600 MPa were assessed when cooked ham was compared to cured meat for risk associated to *Salmonella*, *L. monocytogenes*, and *S. aureus* (Jofré, Aymerich, Monfort, et al., 2008). The decrease of *L. monocytogenes* counts was higher for cooked than cured meat products and for nisin than enterocins A and B and sakacin. *Salmonella* was not affected by the bacteriocins. After 7 days of storage at 4°C, absence of both pathogens was achieved in all batches and kept until the end of storage, even in abusive temperature (15°C). For *S. aureus*, reductions were lower and only the application of nisin in dry-cured ham produced a significant reduction in the counts. Thus, at the end of storage, while *S. aureus* counts were <1 log CFU/g in all dry-cured ham batches, only nisin, as previously reported, could inhibit its growth in cooked ham. Cooked and dry-cured meat products have a similar pH but diferent a_w. Water activity is recognized as playing an important role on HHP inactivation.

A different and promising way to apply antimicrobials is active packaging, an innovative concept that could be defined as a packaging system where the pack, the product, and the environment interact and change the condition of packed food, extending the shelf-life and improving the food safety or the sensorial properties of the product thus preserving its quality (European Commission, 2004; Suppakul, Miltz, Sonneveld, & Bigger, 2003; Vermeiren, Devlieghere, & Debevere, 2002). Application of enterocins A and B, sakacin K, nisin A, potassium lactate, and nisin plus lactate as interleaver together with an HHP treatment at 400 MPa was also evaluated in sliced cooked ham (Jofré et al., 2007; Jofré, Aymerich, & Garriga, 2008). HHP produced an important reduction in *Salmonella*; however, the elimination of the pathogen was only achieved when nisin was absorbed in the interleaver. The interleaver application of nisin had a more long-lasting effect on *L. monocytogenes* than through its application to meat batter (4.5 log CFU/g at the end of 3-month storage when applied in the meat batter and only 1.2 log CFU/g when applied in the interleaver). On the contrary, potassium lactate was much more efficient when applied in meat batter than through interleavers (Fig. 7.3). Therefore, the antimicrobial complement of HHP treatment may depend on its application form and refrigerated storage. It is important to consider this when several hurdles must be applied in order to obtain value-added ready-to-eat products with a safe long-term storage. A further synergistic effect of the bacteriocins enterocins and HHP (400 MPa) against *L. monocytogenes* was observed when the antimicrobials were applied in biodegradable active packaging such as alginate films (Marcos et al., 2008). Three lots of cooked ham were prepared: control, packaging with alginate films, and packaging with antimicrobial alginate films containing enterocins (2000 AU/cm^2). After packaging, half of the samples were pressurized. Sliced cooked ham stored at 6°C experienced a quick growth of *L. monocytogenes*. Both antimicrobial packaging and pressurization delayed the growth of the pathogen. However, at 6°C the combination of antimicrobial packaging and HPP was necessary to achieve a reduction of inoculated levels without recovery during 60 days of storage. Further storage at 6°C of pressurized

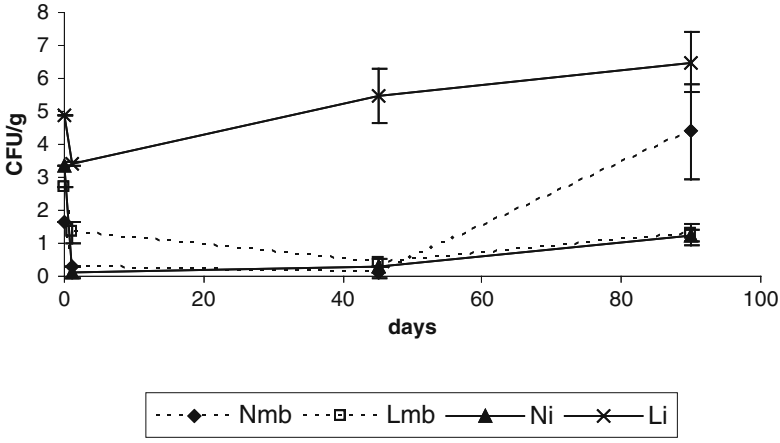

Fig. 7.3 Differential effect of antimicrobial application modi (nisin and lactate) against *L. monocytogenes* together with an HHP treatment of 400 MPa 10 min 17°C
N, nisin; L, lactate; mb, meat batter; i, interleaver.

antimicrobial-packed cooked ham resulted in *L. monocytogenes* levels below the detection limit (day 90). On the other hand, storage at 1°C controlled the growth of the pathogen until day 39 in non-pressurized ham, while antimicrobial packaging and storage at 1°C exerted a bacteriostatic effect for 60 days. All HPP lots stored at 1°C kept the levels of 0.60 log CFU/g achieved after treatment after day 60 (Fig. 7.4). After a cold chain break no growth of

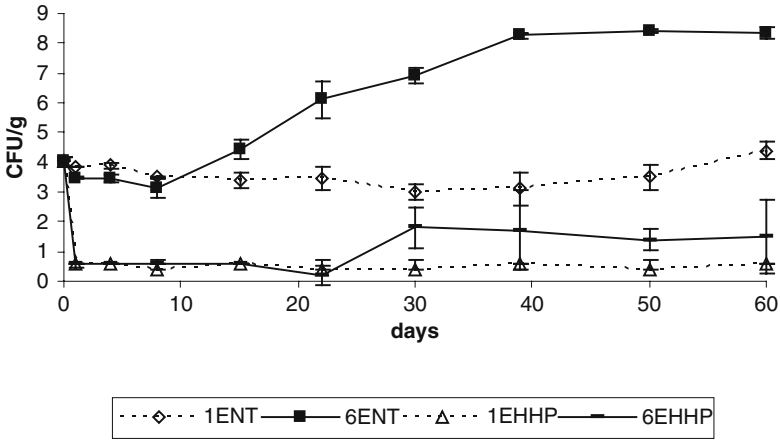

Fig. 7.4 Importance of the hurdle technology on *L. monocytogenes* behavior in sliced cooked ham stored in refrigeration for 60 days
E and ENT-enterocins-alginate film (2,000 AU/cm^2). 1ENT, enterocins 1°C; 6ENT, enterocins 6°C; 1EHHP, enterocins, HHP (400 MPa) 1°C; 6EHHP, enterocins, HHP (400 MPa) 6°C.

L. monocytogenes was observed in pressurized ham packed with antimicrobial films, showing the higher efficiency of combining both technologies (Marcos et al., 2008). In this system the microbial substance would gradually migrate from the pack (container) to the food through diffusion and partitioning or release through evaporation in the headspace during storage and distribution, thus being able to reduce the post-processing contaminations in the surface of the ready-to-eat products during storage (Han, 2005). Combination of active packaging with HPP may thus be considered as an alternative technique to increase the efficiency of these natural antimicrobials whose activity could be reduced by interaction with the food matrixes. When applied with biodegradable film, the technology could be even more environmentally friendly.

The combination of starter culture and HPP after ripening was recommended to produce low-risk and high-quality slightly fermented sausages (Garriga et al., 2005; Marcos et al., 2007). Starter cultures were able to control the growth of *L. monocytogenes*, *S. aureus*, *Enterobacteriaceae, Enterococcus*, and the biogenic amine content. HHP treatment (400 MPa) promoted an additional reduction of *Enterobacteriaceae* and *L. monocytogenes* counts and it was crucial to assess the absence of *Salmonella* spp. While starter cultures slightly modified the pH values and cohesivenes in *fuet* and increased in cohesiveness, springiness, and chewiness but no change in TBARS or color parameters was observed after HHP.

Some other antimicrobials as TBHQ (100–300 ppm) and nisin (100 and 200 ppm) were reported in combination with HHP (600 MPa, 28°C) (Chung et al., 2005) in inoculated commercial sausage samples with 10^6–10^7 CFU/g barotolerant *L. monocytogenes*. Most of the samples treated with nisin, TBHQ, or their combination were positive for *L. monocytogenes*. HPP alone resulted in a modest decrease in the number of positive samples, but *L. monocytogenes* was not detected in any of the inoculated commercial sausage samples after treatment with HPP-TBHQ or HPP–TBHQ–nisin combinations. These results suggest that addition of TBHQ or TBHQ plus nisin to sausage followed by in-package pressurization is a promising method for producing *Listeria*-free RTE products.

Hurdle technology may also be applied to improve physico-chemical and sensorial properties. While raw chicken meat is oxidatively stable, HPP at 600 MPa and above induces lipid oxidation, resulting in off-flavors during subsequent cooking due to the presence of highly unsaturated fatty acids and low tocopherol content. Addition of 0.1% dried rosemary to minced chicken thighs or breasts prior to HPP inhibits lipid oxidation during subsequent cooking and could form the basis for product development (Bragagnolo, Danielsen, & Skibsted, 2007). Sage was also found to protect minced chicken breast processed with HPP up to 800 MPa for 10 min against lipid oxidation during subsequent chilled storage for 2 weeks. Garlic showed prooxidative additive effects especially at moderately high pressure around 300 MPa, an effect partly counteracted by simultaneous addition of sage (Mariutti, Orlien, & Bragagnolo, 2008).

Final Conclusions

High hydrostatic pressure is an alternative and industrially attractive (600 MPa for 5 min) non-thermal technology with higher potential of application to meat products. The technology may be used for hygienization of sliced cooked or cured meat products extending its shelf-life without major changes in sensorial properties. Nevertheless raw products are not so well suited to pressure treatment. HPP at low temperatures, i.e., frozen fresh meat is under study in order to avoid non-desired textural modifications. Moreover, the addition of several natural additives is being considered in order to avoid oxidative reaction as in poultry. However, more in-depth studies must be carried out in order to predict the microbial resistance and the role of bacterial stress during the shelf-life of the product. The treatment must be optimized and accurately defined with a view to legislation. Different stakeholders must interact to convince consumers of their convenience with objective and unbiased data including negative aspects and limitations. Nevertheless, as cells need adaptation before their growth, extension of their lag time by a combination of storage temperature–-high-pressure treatment will result in a considerable delay before spoilage, and hence, the shelf-life before opening the package will be extended. Thus, and according to Smelt and Hellemons (1998) sublethal injury of microorganisms could also be a tool in food preservation. Moreover, the results of different investigations suggest that addition of different antimicrobials followed by in-package pressurization may improve the efficiency of the treatment and could be considered a good strategy to produce food-borne pathogen-free RTE products.

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Chapter 8
Advanced Decontamination Technologies: Irradiation

Eun Joo Lee and Dong U. Ahn

Introduction

Bacterial food-borne illnesses account for an estimated 76 million cases, 325,000 hospitalizations, and 5,000 deaths each year in the United States (CDCP, 2005), and 5,300 food-borne outbreaks in Europe resulted in 5,330 hospitalizations and 24 deaths in 2005 (Aymerich, Picouet, & Monfort, 2008). Major food-borne pathogens of concern include *Escherichia coli* O157:H7, *Campylobacter jejuni/coli*, *Salmonella* spp., *Listeria monocytogenes, Clostridium botulinum/perfringens, Staphylococcus aureus, Aeromonas hydrophylia*, and *Bacillus cereu*, and spoilage microorganisms include *Pseudomonas, Acinetobacter/Moraxella, Aeromonas, Alteromonas putrefaciens, Lactobacillus*, and *Brochothrix thermosphecta* (Mead et al., 1999).

Meat is one of the major foods that cause food-borne illness in human and thus meat sanitation systems are required to use various intervention strategies to reduce or eliminate bacteria. Preharvest reduction of microorganisms in livestock and postharvest decontamination of carcass and meat are common intervention strategies for pathogens in meat (Ahn, Lee, & Mendonca, 2006). Intensifying the immune system of live animals by dietary supplementation of known immune stimulants is commonly used as a preharvest intervention. Postharvest intervention methods of meat use various chemical and physical treatments, which include carcass decontamination, antimicrobial additives, and irradiation.

Irradiation is among the most effective postharvest intervention methods for inactivating food-borne pathogens in meat. Exposing meat products under ionizing radiation such as gamma rays or high-energy electrons can kill pathogens as well as indigenous microflora, and extend shelf life. The major advantages of irradiating foods include (1) potentially toxic chemicals can be avoided and (2) products can be treated after final packaging, and thus, further cross

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contamination during postprocessing handling is prevented. The toxicological and microbiological evaluations of irradiation as well as wholesomeness for irradiated foods have been studied for over 60 years (WHO, 1994), and no other food technology has such a long history of scientific research before gaining approval (AMA, 1993).

Food Irradiation

Since Willhelm von Roentgen discovered X-rays in 1895, the use of ionizing radiation to preserve foods by destroying spoilage microorganisms was proposed (Minsch, 1896; Brynjolfsson, 1989) and X-rays were applied to kill *Trichina* in pork in 1921 (Schwartz, 1921). However, food irradiation was economically unfeasible in the United States until World War II because of the high cost of ionizing radiation sources (Urbain, 1989). The Department of the Army, the Atomic Energy Commission, and private industry sponsored the exploratory food irradiation research in the United States from 1940 to 1953 (Thayer, Lachica, Huhtanen, & Wierbicki, 1986). Since the US Army Medical Department began to assess the safety of irradiated foods in 1955 (CAST, 1986), petitions for the approval of irradiation of specific foods to the Food and Drug Administration (FDA) were followed and commercial radiation equipments and sources were developed. The food irradiation facility for the Army's research laboratories was built in Natick, Massachusetts, in 1962 and the Army conducted scientific food irradiation researches using bacon, ham, pork, beef, hamburger, corned beef, pork sausage, codfish cakes, and shrimp. In 1963, wheat and wheat powder were the first products approved by the FDA (Mason, 1992; Federal Register, 1999). The National Aeronautics and Space Administration (NASA), a pioneer in the use of irradiated food, first used irradiated meats in 1972 for astronauts to consume in space and then irradiated ham, turkey, beef steak, and corned beef were used in the Apollo–Soyuz Test Project (ASTP) where irradiated foods were shared with the Russian cosmonauts in 1975 (Karel, 1989).

In 1980, the Food and Agriculture Organization of the United Nations, the International Atomic Energy Agency, and the World Health Organization (FAO/IAEA/WHO) stated that "irradiation of any food commodity up to an overall average dose of 1 Mrad (10 kGy) presents no toxicological hazard and introduces no special nutritional or microbiological changes; hence toxicological testing of foods so treated is no longer required" (WHO, 1981). During 1980s, the FDA approved petitions for irradiation of spices and seasonings, fresh fruits, and dry substances and the USDA-approved pork (USDA-FSIS, 1986), poultry (USDA-FSIS, 1992), and red meats (USDA-FSIS, 1999). The use of irradiation in meat is restricted to raw, packaged poultry at 1.5–3.0 kGy, and fresh and frozen red meat at a maximum dose up to 4.5 and 7.0 kGy, respectively (Sommers, 2004). The maximum irradiation doses approved for

Table 8.1 Maximum irradiation dose

Year approved	Food	Dose (kGy)	Purpose
1963	Wheat flour	0.2–0.5	Control molds
1986	Fresh fruit and vegetables	1.0	Inhibit sprouting, delay ripening, disinfestation of insects
1986	Dehydrated spices and herbs	30	Control pathogens
1990	Poultry meat	3.0	Control pathogens
1999	Refrigerated meat	4.5	Control pathogens
	Frozen meat	7.0	Control pathogens
	Dehydrated enzymes	10	Control pathogens
2000	Shell eggs	3.0	Control of *Salmonella*
2005	Molluscan shellfish	5.5	Control of *Vibrio* bacteria and other food-borne microorganisms

food products in the United States are listed in Table 8.1. Currently, 56 countries have permitted irradiation of food, and more than half a million tons of food are irradiated annually in the world (Loaharanu, 1994; NAPPO, 1995; IAEA, 1999)

Principles of Irradiation

Atoms are consisted of protons, neutrons, and electrons, which are held together by energy (Thakur & Singh, 1994). When these nuclear particles lose balance by changing the arrangement of forces, this unstable atom can restabilize by emitting energy to rebalance the nucleus. This emission of energy, as particles or waves, is termed "radiation" (Halliwell & Gutteridge, 1989). If radiation has sufficient energy to move atoms in another material without chemical changes it is called as a "non-ionizing radiation", and if it also has sufficient energy to break chemical bonds it is called as "ionizing radiation" (Josephson & Peterson, 2000). High-energy sources such as accelerated electrons, gamma rays, and X-rays are ionizing radiations because those can create ions or free radicals from atoms (CAST, 1989).

Gamma rays are emitted as a photon from nucleus and X-rays from electron fields when the energy of atoms is exhausted (Lagunas-Solar, 1995). Gamma rays do not ionize atoms directly; when a photon or an accelerated electron enters a material, the electron of an atom in that material increases in energy level and leaves its orbit. The ejected electron, called "Compton electron", transfers its energy to a secondary electron and cause further excitation and ionization in the material (Diehl, 1995). Until Compton electron's energy is not enough to cause electrons to leave their orbital, energy is passed through a

cascade of electrons (Venugopal, Doke, & Thomas, 1999). The quantity of energy absorbed by something (food) as it passes through a radiation field is called "radiation absorbed dose". The unit of irradiation dose is expressed as Gray (Gy), which is equal to the absorption of energy equivalent to one Joule per kilogram of absorbing material (1 Gy = 1 J kG^{-1} = 6,200 billion MeV absorbed/kg of food = 0.01 calorie/lb of food = 100 rad, 1 rad = 100 erg/g) (Dragnic & Dragnic, 1963).

As irradiation energy applied to biological materials ejects electrons from the atoms or molecules of the materials and produces ions and free radicals (Woods & Pikaev, 1994), the cellular components such as DNA, pigments, fatty acids, and membrane lipids can be damaged (Olson, 1998a). The first target of highly energized electrons is water molecule in biological materials and hydroxyl radical (HO•), a powerful oxidizing agent, is formed (Taub, Karielian, & Halliday, 1978; Taub et al., 1979). The dispersion of free radicals is higher in liquid form than in limited free water form (dried products) or the crystalline form (frozen products) (Thakur & Singh, 1994). When the DNA of living cells is exposed to hydroxyl radicals, both single and double strands in the molecule are broken and large molecules have a greater probability of being affected than smaller molecules because dispersion of electrons are purely random. Therefore, human have a greater damage than microorganism when they are exposed to radiation energy and higher dose of radiation energy is required to kill microorganism than bigger size animals (Thayer, 1995).

Currently, both e-beam and gamma rays are used as radiation sources for commercial food irradiation. ^{60}Co is the most common energy source that produces gamma rays and commonly used to treat food contained inside a package because it is highly penetrable (Venugopal et al., 1999). Electron beam is a stream of high-energy electrons that are propelled out of an electron gun (Josephson & Peterson, 2000). Electron beam accelerators accelerate electrons to a beam (up to 10 MeV) with single-sided treatment and 10 MeV electrons can give satisfactory treatment for thicknesses up to about 35 mm of unit density material. Using a conveyor belt with double-sided, a product thickness of 8 cm can be used (Satin, 2002). Although electrons are less penetrable than gamma rays, electron beam can be useful for large volumes of free flowing food items such as grains or packages of fish fillets with no more than 8–10 cm thickness (Jarrett, 1982). Although X-rays have relatively high penetrating power, they are rarely used in food irradiation due to poor conversion of accelerated electrons to X-rays (Hayashi, 1991).

Microcidal Effect of Irradiation

Several factors such as irradiation dose, meat composition, temperature, gaseous atmosphere, and microbial factors influence to kill microorganisms in meats by irradiation (Olson, 1998b). High doses of radiation are needed to

destroy larger populations of food-borne microorganism, but it negatively impacts the organoleptic qualities of meat. High amount of proteins and natural antioxidants such as carnosine and vitamin E in meat decrease the antimicrobial efficacy of ionizing radiation because they neutralize free radicals (Diehl, 1995; Steccheni et al., 1998). Irradiation of sweeteners such as dextrose produces peroxides, which theoretically should further contribute to microbial inactivation during irradiation of dextrose-containing RTE meats (Kawakishi, Okumura, & Namki, 1971). Free fatty acids, carbonyl compounds, hydrogen peroxide, and hydroperoxides produced from irradiated fats increase the killing effect of irradiation in foods (Diehl, 1995).

Freezing meat reduces water activity by converting water to ice. The reduced water activity increases irradiation resistance of microorganisms because the generation of free radicals from water is drastically reduced (Diehl, 1995) and the migration of free radicals to other parts of the frozen product is impeded (Taub et al., 1979). Most published research indicated that the presence of oxygen increased the killing effect of irradiation in meat (Hastings, Holzapfel, & Niemand, 1986; Patterson, 1988; Fu, Sebranek, & Murano, 1995; Thayer & Boyd, 1999).

Microbial factors such as numbers, types, and physiological status of microorganisms in meat can affect the extent of microbial destruction by irradiation. For example, viruses have much higher radiation resistance than bacterial

Table 8.2 D-values of food-borne pathogens and spoilage bacteria

Pathogen	D_{10} (kGy)	Medium	References
A. hydrophila	0.14–0.19	Beef	Palumbo et al. (1986)
B. Cereus (vegetative)	0.17	Beef	Grant et al. (1993)
C. jejuni	0.08–0.20	Beef	Clavero et al. (1994)
C. perfringens	0.59–0.83		Farkas (2006)
E. coli O157:H7(incl. 0157H7)	0.23–0.35	Beef	Clavero et al. (1994)
Lactobacillus spp.	0.3–0.9		Farkas (2006)
L. monocytogenes	0.42–0.55	Chicken	Huhtanen et al. (1989)
	0.57–0.65	Pork	Grant and Patterson (1991)
	0.51–059	Beef	Monk et al. (1994)
Salmonella spp.	0.38–0.80	Chicken	Thayer et al. (1991)
Staphylococcus aureus	0.0.26–0.6	Chicken	Thayer and Boyd (1992)
	0.39	Roast beef	Patterson (1988)
Y. enterocolitica	0.04–0.21	Beef	El-Zawahry and Rowley (1979)
Cl. botulinum (spore)	3.56	Chicken	Anellis et al. (1977)
C. sporogenes (spore)	6.3	Beef fat	Shamsuzzaman and Lucht (1993)
M. phenylpyruvica	0.63–0.88	Chicken	Patterson (1988)
P. putida	0.08–0.11	Chicken	Patterson (1988)
S. faecalis	0.65–1.0	Chicken	Patterson (1988)

spores, which in turn show a higher radiation resistance than bacterial vegetative cells. Bacterial vegetative cells are more radiation resistant than fungi (yeast and molds). Gram-negative bacteria are generally more sensitive to ionizing radiation than Gram-positive bacteria (Ehioba et al., 1988; Lambert, Smith, & Dodds, 1992; Thayer, Boyd, & Jenkins, 1993). Non-spore forming bacteria exhibit a greater sensitivity to irradiation than spore formers. The radiation response of microbial populations is expressed by the decimal reduction dose (D_{10}-value), and the D_{10}-values of food-borne pathogens and spoilage bacteria are listed in Table 8.2

Effects of Irradiation on Meat Quality

The application of irradiation technology in meat industry is limited because of quality and health concerns about irradiated meat products. Irradiation produces a characteristic aroma and color that significantly impact upon consumer acceptance. Consumers associate the brown/gray color in raw beef with old or low-quality meat, red/pink color in irradiated cooked light meat with undercooked or contaminated, and off-odor and off-flavor with undesirable chemical reactions. Thus, developing methods that can prevent these quality changes in meat by irradiation is important for implementing irradiation technology by the meat industry.

Ionizing radiation generates hydroxyl radicals, the most reactive oxygen species in nature, by splitting water molecules (Thakur & Singh, 1994). Thus, irradiation can increase lipid oxidation in meat significantly because meat contains 75% or more of water. The presence of oxygen also has a significant effect on the development of lipid oxidation and odor production (Merritt, Angelini, Wierbicki, & Shuts, 1975). Therefore, excluding oxygen from meat products, whether they are irradiated or not, is very important to stop oxidative chain reactions (Ahn, Wolfe, Sim, & Kim, 1992). Ahn et al. (1998) reported that preventing oxygen exposure after cooking was more important for cooked meat quality than packaging, irradiation, or storage conditions of raw meat. Diehl (1999) indicated that irradiation of aqueous systems produced hydrogen peroxide, particularly in the presence of oxygen. During postirradiation storage, hydrogen peroxide gradually disappears while other constituents of the system are oxidized. Lee and Ahn (2003) reported that TBARS values of oil emulsion samples immediately after irradiation were lower than those of nonirradiated samples. After 10 days of storage, however, irradiated samples developed higher TBARS values than nonirradiated emulsions. Especially arachidonic acid, linolenic acid, and fish oil, which had a high proportion of polyunsaturated fatty acids, had accelerated lipid oxidation after irradiation. Nawar (1986) reported that a series of dienes, trienes, and tetraenes were formed from unsaturated triacylglycerols by irradiation at 60 kGy under vacuum conditions. The radiation chemistry of refrigerated and frozen meat could

be different because free radicals with less mobility in the frozen state tend to recombine to form the original substances rather than diffuse through the food and react with other food components (Taub et al., 1979). Therefore, oxidative changes in irradiated frozen products are slower than that of the refrigerated products.

Irradiation greatly increased or newly produced many volatile compounds such as 2-methyl butanal, 3-methyl butanal, 1-hexene, 1-heptene, 1-octene, 1-nonene, hydrogen sulfide, sulfur dioxide, mercaptomethane, dimethyl sulfide, methyl thioacetate, dimethyl disulfide, and trimethyl sulfide from meat (Ahn, Jo, & Olson, 2000; Fan, Sommers, Thayer, & Lehotay, 2002). Early investigators assumed that the off-odor was the result of lipid oxidation, which was initiated by the irradiation process. They postulated that carbonyls were formed in irradiated meats after the reactions of hydrocarbon radicals, the major radiolytic products in fat, with molecular oxygen (Champaign & Nawar, 1969; Merritt, Angelini, & Graham, 1978) and it caused the off-odor of irradiated meat with following the same pathway as normal lipid oxidation. Aldehydes contributed the most to oxidation flavor and rancidity in cooked meat and hexanal was the major volatile aldehyde (Shahidi & Pegg, 1994). When triglycerides or fatty acids are irradiated, hydrocarbons are formed by cutting CO_2 and CH_3COOH off from fatty acids in various free radical reactions (Morehouse, Kiesel, & Ku, 1993).

However, sensory results clearly indicated that the main source of irradiation off-odor was caused by sulfur compounds. All irradiated meat develop a characteristic odor, which has been described as "metallic", "sulfide", "wet dog", "wet grain", or "burnt" (Huber, Brasch, & Waly, 1953), "bloody and sweet" (Hashim, Resurreccion, & MaWatters, 1995), "hot fat," "burned oil," or "burned feathers" (Heath, Owens, Tesch, & Hannah, 1990), and "barbecued corn-like" (Ahn, Olson, Jo, Love, & Jin, 1999). The odor intensity of sulfur compounds was much stronger and stringent than that of other compounds because most sulfur compounds have very low-odor thresholds (Lee & Ahn, 2003). Therefore, sulfur compounds would be the major volatile components responsible for the characteristic off-odor in irradiated meat, and volatiles from lipids accounted for only a small part of the off-odor in irradiated meat (Angelini, Merritt, Mendelshon, & King, 1975).

To support the sulfur theory for off-odor production in irradiated meat, studies were conducted using various amino acid homopolymers (Ahn, 2002; Ahn & Lee, 2002). The results indicated that the sulfur compounds produced from irradiated methionine and cysteine had an odor characteristic similar to that of irradiation odor of meat. Sulfur-containing amino acids such as methionine and cystein were the major sources of sulfur volatiles upon irradiation, but the amount of sulfur compounds from cystein was only about 0.25–0.35% of methionine. Therefore, the contribution of methionine to the irradiation odor was far greater than that of cysteine. Other studies on the volatile profiles and sensory characteristics of amino acids clearly indicated that irradiation odor was different from lipid oxidation odor, and lipid

oxidation was responsible for only a small part of the off-odor in irradiated meat (Ahn et al., 1999, 2000, 1998).

Mechanisms related to the radiolysis of amino acids are not fully understood, but deamination during irradiation is one of the main steps involved in amino acid radiolysis (Dogbevi, Vachon, & Lacroix, 1999). The degradation of amino acids by oxidative deamination–decarboxylation via the Strecker degradation produces branched chain aldehydes (Mottram, Wedzicha, & Dodson, 2002), which may be the mechanism for the formation of 3-methyl butanal and 2-methyl butanal during irradiation from leucine and isoleucine, respectively (Jo & Ahn, 2000). Davies (1996) reported that irradiation of *N*-acetyl amino acids and peptides in the presence of oxygen gives high yields of side chain hydroperoxides, which can be formed on both the backbone (at alpha-carbon positions) and the side chains. The interactions among food components such as carbohydrates, lipids, and proteins (Godshall, 1997), and the physicochemical conditions of foods, which influence conformation of proteins, also affect the release of volatile compounds in foods (Lubbers, Landy, & Voilley, 1998). This indicated that the relative amounts of volatile compounds released from meat systems could be significantly different from those of the aqueous model systems (Jo & Ahn, 2000).

The color changes in irradiated meat vary significantly depending on various factors such as irradiation dose, animal species, muscle type, and packaging type (Shahidi, Pegg, & Shamsuzzaman, 1991; Luchsinger et al., 1996; Nanke, Sebranek, & Olson, 1999). Generally, irradiation increased redness of light meat such as poultry breast and pork loin (Nam & Ahn, 2002b; Millar, Moss, MacDougall, & Stevenson, 1995; Nam, Ahn, Du, & Jo, 2001), and changes the red color of beef to greenish brown under aerobic conditions, which would be unattractive to consumers (Nam & Ahn, 2003b). Early investigators assumed that the bright red color in light meat after irradiation was oxymyoglobin formed by the reaction between metmyoglobin and hydroxyl radicals (Tappel, 1956). Oxymyoglobin was formed by the reduction of heme iron by a radiolytic water product, hydrated electron, and the oxygenation from either residual oxygen or generated oxygen during irradiation (Giddings & Markakis, 1972). However, it is very difficult to accept that the pigment as an oxymyoglobin because the red color formed by irradiation has been produced mainly in anoxic conditions. Millar et al. (1995) postulated that the red/pink color in irradiated light meat was due to a ferrous myoglobin derivative such as carboxyl-myoglobin or nitric oxide-myoglobin other than oxymyoglobin. Nam and Ahn (2002a, 2002b), however, suggested that carbon monoxide–myoglobin (CO–Mb) caused the pink color in irradiated light meat. Considerable amounts of carbon monoxide were produced from organic components such as alcohols, aldehydes, ketones, carboxylic acids, amides, and esters by irradiation (Woods & Pikaev, 1994; Furuta, Dohmaru, Katayama, Toratoni, & Takeda, 1992). Lee and Ahn (2004) reported that glycine, asparagine, glutamine, pyruvate,

glyceraldehydes, α-ketoglutarate, and phospholipids were the major sources of CO production among meat components by irradiation.

The decrease of ORP in meat played a very important role in CO–Mb formation because the CO–Mb complex can only be formed when heme pigment is in reduced form (Cornforth, Vahabzadeh, Carpenter, & Bartholomew, 1986). Irradiation lowered oxidation–reduction potential (ORP) in light meat, but the ORP in irradiated meat increased rapidly during storage under aerobic conditions while maintained under vacuum-packaging conditions (Nam & Ahn, 2002b). This result indicated that the increase of ORP facilitated the conversion of myoglobin from ferrous to ferric form, which reduced the affinity of CO to heme pigments, and thus, reduced pink color intensity in meat upon exposure to air. Although, the affinity of CO to Mb is 200-fold higher than that of oxygen (Stryer, 1981), the concentration of oxygen in atmosphere is much higher than that of CO. Therefore, continuous challenge of oxygen under aerobic conditions eventually replaces or removes all CO from heme pigments and reduces the intensity of pink color. In conclusion, three essential factors – production of CO, generation of reducing conditions, and CO–Mb ligand formation – cause the pink color formation of light meats by irradiation. In addition, light meat had higher ferric iron-reducing power than red meat, which facilitated the reduction of heme pigments (Min, Nam, Cordray, & Ahn, 2008).

The mechanisms of color change in irradiated red meat such as beef are different from those of light meats, and the proposed color-changing mechanisms in irradiated beef are as follows: irradiation produces aqueous electrons (e_{aq}-) and hydrogen radicals that have reducing power from water molecules (Thakur & Singh, 1994). Thus, in the absence of O_2, a reducing environment is established and all the heme pigments in beef are in ferrous form and color is red (Satterlee, Wilhelm, & Barnhart, 1971). In the presence of oxygen, however, strong-oxidizing agents (superoxide and hydroperoxyl radicals) are formed from the reactions of O_2 and e_{aq}- and O_2 and H, respectively (Giddings, 1977). Therefore, irradiation under aerobic conditions favors ferric Mb (brown color) but produces ferrous Mb (red color) under vacuum conditions. The content of heme pigments in beef is about 10-times greater than that of light meats, and the proportion of carbon monoxide–myoglobin (CO–Mb), the compound responsible for color changes in irradiated light meats, to total heme pigments in irradiated beef is small. Therefore, overall beef color is mainly determined by the status of heme pigments, which is determined by the reducing potential of meat. Green pigment in irradiated beef was formed by hydrosulfide produced from glutathione or thiol-containing compounds (Fox & Ackerman, 1968).

Irradiation may produce nitric oxide or other precursors to the cured meat pigment, nitrosyl hemochrome, particularly if nitrite or nitrate ions are present (Cornforth et al., 1986). Nitric oxide radical can be generated from nitrogen-containing amino acid side chains (e.g., arginine, glutamine) by an oxidative stress such as irradiation (Thomas, 1999). Packaging environment is an important factor that influences the color of irradiated meat during storage.

Irradiation increased the a*-value (redness) of both aerobically and vacuum-packaged light meats, but the latter was significantly redder than the former during storage (Luchsinger et al., 1997; Nanke et al., 1999; Nanke, Sebranek, & Olson, 1998). Sensory panelists preferred the red color of irradiated light meats to nonirradiated ones because they looked fresh (Lefebvre, Thibault, Charbonneau, & Piette, 1994). However, increased redness in irradiated meats can be a problem if the red color persists in meat after cooking.

Irradiation significantly increased centrifugation loss of water from pork loins (Zhu, Mendonca, & Ahn, 2004) because of the damage in the integrity of membrane structure of muscle fibers (Lakritz, Carroll, Jenkins, & Maerker, 1987) and the denaturation of muscle proteins, which reduced water-holding capacity (Lynch, Macfie, & Mead, 1991) by irradiation. Transmission electron microscopy showed significant differences in size of myofibril units (sarcomeres) between irradiated and nonirradiated breasts (Yoon, 2003). Lewis, Velasquez, Cuppett, and McKee (2002) found that the texture attributes were lower in irradiated chicken breasts. However, others reported that irradiation had minimal effects on texture of frozen, raw and precooked ground beef patties (Fu et al., 1995), frozen and chilled boneless beef steaks (Luchsinger et al., 1997), and RTE turkey breast rolls (Zhu, Mendonca, Min, et al., 2004).

Consumer acceptance of irradiated meat is important to adopt irradiation technology by meat industries (AMIF, 1993). Despite years of efforts to introduce irradiated foods to marketplace, many consumers still misunderstand the effectiveness, safety, and functional benefits of irradiation technology (Fox, Hayes, & Shogren, 2002). Consumers' willingness to buy irradiated foods varied depending on gender, education level, income, geographic location, and exposure to irradiated food products (Frenzen et al., 2001). The proportion of consumers buying irradiated meat increased after the participants of study received additional information about food irradiation (Hashim et al., 1995), indicating that consumers' knowledge about irradiated foods is among the most important factors for the acceptance of irradiated foods (Lusk, Fox, & McIlvain, 1999).

Prevention of Quality Changes in Irradiated Meat

Many researchers have studied methods to prevent the quality changes of irradiated meat using various additives and packaging types. Antioxidants, such as ascorbate, citrate, tocopherol, gallic esters, and polyphenols, prevented oxidative rancidity, retarded development of off-flavors, and improved color stability in irradiated fresh and further processed meat (Morrissey, Brandon, Buckley, Sheehy, & Frigg, 1997; Xiong, Decker, Robe, & Moody, 1993; Huber et al., 1953). Meat industries prefer to use natural antioxidants such as rice hull extract, sesamol, and rosemary oleoresin because of consumer demands for natural products and those have effective to reduce off-odor volatiles such as

aldehydes and dimethyl disulfide in irradiated turkey meat (Lee, Love, & Ahn, 2003; Nam et al., 2006). Dietary antioxidant treatments for live animal also have shown to reduce the extent of lipid oxidation in irradiated meat during storage (Morrissey et al., 1997; Wen, Morrissey, Buckley, & Sheehy, 1996; Winne & Dirinck, 1996). Lowering pH using acids such as citric and ascorbic acids was expected to decrease redness in irradiated meat because hydroxide anion produced by irradiation was also decreased at low pH status, but it did not affect the redness of irradiated light meat (Nam & Ahn, 2002c). In red meat, however, reducing agent such as ascorbic acid was very effective in maintaining redness and preventing greenish brown discoloration by irradiation (Nam, Min, Park, Lee, & Ahn, 2003). The lowered ORP values by ascorbic acid maintained heme pigments in ferrous status and stabilized the color of irradiated ground beef (Nam & Ahn, 2003b). Addition of antimicrobial agents such as lactate, acetate, and sorbate had synergistic effects with irradiation in killing microorganisms in meat and also had positive effects on the quality of irradiated meat products (Zhu, Mendonca, Min, et al., 2004; Zhu et al., 2005). Therefore, combined use of antimicrobial agents with irradiation can improve the safety of meat products without significant impact on meat quality.

Packaging is an important factor influencing color and volatiles in irradiated meat. Vacuum packaging prevented oxidative changes and color fading in irradiated meat, but retained S-volatiles such as methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide (Nam et al., 2003; Nanke et al., 1999). Aerobic packaging was more desirable for the irradiated meat color, especially in light meat, and off-odor than vacuum packaging if lipid oxidation can be controlled (Ahn, Jo, Olson, & Nam, 2000; Ahn, Nam, Du, & Jo, 2001). Exposing irradiated meat to aerobic conditions for a certain period was helpful in reducing off-color because of competition between atmospheric oxygen and carbon monoxide produced by irradiation and off-odor because of the volatilization of sulfur compounds(Nam & Ahn, 2002a). However, exposing irradiated meats to aerobic conditions increased lipid oxidation (Nam & Ahn, 2003a). Thus, an appropriate combination of aerobic and vacuum packaging, called "double packaging", was effective in reducing the generation of off-odor and off-color in irradiated meat during the storage (Nam & Ahn, 2002c). The term "double packaging" is to describe a packaging method in which irradiated meat pieces are individually packaged in oxygen-permeable bags (aerobic condition) at first and then a few of aerobic packages are packaged again in a larger vacuum bag (Nam & Ahn, 2003a). If the outer vacuum bag is removed after certain storage time and then displayed under aerobic conditions until the last day of storage, it minimized lipid oxidation, off-odor production, and color change (Nam & Ahn, 2003c).

Although double packaging improved the quality changes of irradiated meat significantly, aldehydes such as propanal and hexanal, which coincided with the degree of lipid oxidation (TBARS) were detected more in double packaging than in vacuum packaging alone. Therefore, the combination of double packaging with antioxidants such as gallate, α-tocopherol, sesamol, and ascorbic acid

in irradiated meat was suggested and it was very effective in lipid oxidation as well as reducing off-odor, especially irradiated cooked meat (Nam & Ahn, 2002c, 2003b, 2003c). The combined use of double packaging and ascorbic acid was more effective to irradiated ground beef for maintaining bright red color because irradiating under vacuum condition and added reducing agent was helpful in maintaining low ORP of irradiated beef and caused myoglobin to be remained in a reduced form (Nam & Ahn, 2003b).

Toxicity and Health Concerns

Safety concerns about radiolytic compounds such as furan, 2-alhylcyclobutanones (2-ACB), and acrylamide have been raised, despite the fact that only very small amounts of them are present in the irradiated foods. Furan is an aromatic compound found at low concentrations in many irradiated and nonirradiated foods (Maga, 1979) and is considered as a human carcinogen (NTP, 2004). Generally, furan is formed by the thermal decomposition of carbohydrates such as glucose (Walter & Fagerson, 1968) and ascorbic acid (Tatum, Shaw, & Berry, 1969). Therefore, concerns of furan formation in irradiated meat are limited only to RTE products, which contain sugar and ascorbic acid as ingredients and receiving thermal processing. Fan and Sommers (2006) reported that irradiation does not produce furan in RTE meat and poultry products, although furan was formed in aqueous solutions of ingredients used in irradiated RTE meat products and irradiated juices (Fan, 2005).

Acrylamide is known as "a probable human carcinogen" by the International Agency for Research on Cancer (IARC, 1995) and can be formed by Maillard reaction from asparagine and reducing sugars (Yaylayan & Stadler, 2005). Since the Swedish National Food Administration (2002) reported the amount of acrylamide in commonly consumed baked and fried foods, the formation of acrylamide in foods by irradiation has been studied. Fan and Mastovska (2006) reported that irradiation did not induce acrylamide formation in the mixture of reducing sugar and asparagine, but destroyed it in liquid food products.

The toxicological effect of 2-alhylcyclobutanones (2-ACB) in irradiated food has been controversial for many years. Since LeTellier and Nawar (1972) reported 2-ACB in highly irradiated synthetic triglycerides, numerous studies on the production of 2-ACB in several irradiated foods, such as chicken, pork beef, fish, egg, cheese, mango and rice have been conducted (Stevenson, 1996; Ndiaye, Jamet, Miesch, Hasselmann, & Marchioni, 1999; Stewart, Moore, Graham, McRoberts, & Hamilton, 2000). Fat is known as the major source of 2-ACB production and irradiation of palmitic, stearic, oleic and linoliec acid produces 2-ACBs such as 2-dodecyl (2-DCB), 2-tetradecyl (2-TCB), 2-tetradecenyl (2-TDCB), and 2-tetradecadienyl cyclobutanone (2-TDeCB). Because 2-ACB is not detected in nonirradiated foods, they are also used as markers

for irradiated foods. Raul et al. (2002) reported that rats fed 2-TDCB developed tumors in colon and Delincée and Pool-Zobel (1998) reported that 2-DCB caused DNA damage and cell death. However, the amount of 2-ACBs used to induce toxic effects or mutagenecity in animal were thousands times greater than those found in irradiated foods (Health Canada, 2003), and many other recent studies also indicated that the toxicity of 2-ACBs produced in irradiated foods is very low if any (Sommers & Schiestl, 2004; Gadgil & Smith, 2004).

Further Research Needed

Most of the irradiation studies are done with raw meat because irradiation is not permitted for the meats with additives, further processed or precooked ready-to-eat meat products. Therefore, future studies should be focused on flavor, color, and taste changes in further processed and precooked ready-to-eat meat products by irradiation. Methods to prevent quality changes in irradiated further processed or precooked ready-to-eat meat products should also be developed. Although odor and color are important factors for consumer acceptance of irradiated raw meat, the most important quality parameter for cooked meat is taste because if irradiated meat has undesirable taste, consumers will never choose irradiated meat again. Currently, no information on the mechanisms and causes of taste/flavor changes in irradiated cooked meat is available. Therefore, researches to elucidate the causes and mechanisms of taste changes in irradiated cooked meat, determine the roles of spices and additives on taste/flavor of irradiated processed meat, and develop methods that can control taste/flavor changes in irradiated further processed meat are needed. For both raw and cooked meat products, masking of irradiation odor using additives such as natural herb or spices can be an excellent way to solve off-odor problems in irradiated meat. The effect of those additives on the microcidal efficiency of irradiation also should be determined.

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Chapter 9
Control of Thermal Meat Processing

Carl L. Griffis and Tareq M. Osaili

Thermal Processing

The recent growth of the market for ready-to-eat (RTE) meat and poultry products has led to serious concern over foodborne illnesses due to the presence of pathogens, particularly *Salmonella* spp, *Listeria monocytogenes* and *Escherichia coli* O157:H7 in meat and poultry products. Emphasis has been placed on thermal processing since heat treatment is still considered the primary means of eliminating foodborne pathogens from raw meat and poultry products (Juneja, Eblen, & Ransom, 2001). Inadequate time/temperature exposure during cooking is a contributing factor in food poisoning outbreaks. Optimal heat treatment is required not only to destroy pathogenic microorganisms in meat and poultry products but also to maintain desirable food quality and product yield.

Thermal destruction of pathogens is a time–temperature-dependent process. The time–temperature relationship of the thermal inactivation of pathogens has long been expressed with the concept of decimal reduction time (D-value) and thermal resistance constant (z-value). The D-value is defined as the time required to cause a 90% reduction of the microbial population at a specific temperature. It reflects the tolerance of a pathogen to an increase in heating time at a specific temperature. D-value at each temperature is calculated from the linear regression model between $\log_{10}$ of the bacteria survivors and heating time. The D-value is the negative inverse slope of the survivor curve (Equation 9.1). The z-value is the temperature difference required for the thermal inactivation curve to cause one $\log_{10}$

This chapter summarizes some of the published data on the thermal inactivation of some important foodborne pathogens, *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in meat and poultry products.

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reduction. It is correlated with the tolerance of a specific pathogen to the temperature changes in the product. The z-value is calculated by determining the linear regression between $\log_{10}$ of D-values and temperature. The z-value is the negative inverse slope of the thermal resistance curve (Equation 9.2). To calculate D- and z-values, microbial inactivation has traditionally been assumed to follow first order kinetics; i.e., at a certain temperature, the $\log_{10}$ reduction of bacteria is linear over time (Heldman & Hartel, 1997). This assumption is applicable if (1) the relationship of t{T, N} = g{T}.f{N} describes the thermal inactivation of microbes, where t = heating time at certain temperature (T), N = number of survivors at time t (Kormendy & Kormendy, 1997); (2) the sub-lethality injury phenomenon is ignored; and (3) the heating effect on the microorganisms in the food sample is homogenous (Moats, 1971).

$$D = \frac{\log_{10}(N_0) - \log_{10}(N_t)}{t} \tag{9.1}$$

where,

N_0 is the survivors at time 0.
N is the survivors at time t.

$$z = \frac{\log_{10}(D_0) - \log_{10}(D_T)}{\Delta T} \tag{9.2}$$

where,

D_0 is the decimal reduction time (min) at temperature T_0 (°C).
D is the decimal reduction time at temperature T
ΔT is the difference between T and T_0 (T–T_0)
z is thermal resistant constant (°C).

Efforts have been increasing to prevent foodborne illnesses and make the food supply safe. The USDA-FSIS (1999) issued a final rule that requires each processing schedule in a meat or poultry industry to achieve 6.5-D reduction in the population of a cocktail of *Salmonella* serotypes in RTE beef products and 7-D reduction of a cocktail of *Salmonella* serotypes in fully cooked poultry products. The regulation applies to high heat-resistant strains as well as strains that have been implicated in foodborne outbreaks. Thus, thermal inactivation parameters (D- and z-values) for a certain microorganism are necessary to calculate process lethality (F).

$$F = \int_0^t 10^{\left(\frac{T(t) - T(\mathrm{ref})}{z}\right)} \mathrm{d}t$$

where,

$T(t)$ is the product temperature at a time t and T (ref) is a reference temperature. This equation makes clear that z-value should be known in order to calculate the process lethality during cooking. The regulation can be met if the time obtained from the process lethality is equal to or more than the performance standard.

Thermal Inactivation Studies

Most thermal inactivation studies of foodborne pathogens in meat and poultry products have been conducted in metal or glass tubes or in plastic bags. In tubes, the destruction of microorganisms at high temperature is achieved in the outer part of the sample close to the tube wall, but high temperatures do not reach the center of the tubes, and therefore, a majority of the sample does not reach the desired temperature (Orta-Ramirez, 2002). To produce isothermal destruction conditions and more homogeneous heat transfer to the sample, meat samples can be placed in aseptic plastic bags (Juneja, Eblen, & Ransom, 2001; Murphy, Beard, Martin, Duncan, & Marcy, 2004; Osaili et al., 2007). The thickness of the plastic bags has an effect on the thermal inactivation of pathogens in the food sample in the bag. Increasing the thickness of the plastic bag reduces the thermal inactivation rate of pathogens (Murphy, Duncan, Marcy, Berrang, & Driscoll, 2002).

Although thermal destruction of pathogens in food is assumed to be linear with time, deviations from linear destruction with time have been observed by some researchers. These reports indicate that survival curves exhibit a shoulder or tailing. Shouldering phenomena may be related to the ability of treated microorganism to repair the damage at early stages of the exposure to heat. Senhaji and Lincin (1977) reported that the tailing phenomenon shows a variation in thermal resistance within the bacterial strains or accumulation of protective substances released from the destroyed cells.

Factors Affecting Thermal Inactivation of Pathogens in Meat and Poultry Products

Product's Characteristics

Intrinsic properties of the product have an influence on the heat resistance of pathogens associated with the product. Researchers have found varying results concerning the effect of fat content on thermal inactivation of pathogens in meat and poultry products. Some found that fat decreased the thermal resistance of pathogens (Juneja & Eblen, 2000; Kotrola, Conner, & Mikel, 1997), some reported that fat had no effect (Kotrola & Conner, 1997) and most found that fat increased the thermal resistance (Ahmed, Conner, & Huffman, 1995;

Fain et al., 1991; Juneja, Eblen, & Marks, 2001; Line et al., 1991; Maurer, Ryser, Booren, & Smith, 2000). Juneja and Eblen (2000) found that the *D*-values of a cocktail of eight strains of *Salmonella* Typhimurium DT104 in ground beef decreased with increasing fat content. Kotrola and Conner (1997) found increasing the fat level from 3 to 11% in ground turkey did not have a significant effect on the *D*-value of *E. coli* O157:H7 heated at 52.5°C, 55°C, and 60°C. Maurer et al. (2000) reported that *D*-values of *Salmonella* Senftenberg in turkey thighs at temperatures of 58–64°C increased as the fat content increased from 5.3 to 7.4%. Fain et al. (1991) found that fat had a protective effect against *L. monocytogenes* Scott A in ground beef at temperatures of 51.7–62.8°C. They reported *D*-values ranged from 81.3 to 0.6 min, and 71.7 to 1.2 min for lean (2%) and fatty beef (30.5%), respectively. Line et al. (1991), Ahmed et al. (1995) and Smith, Maurer, Orta-Ramirez, Ryser, and Smith (2001) have studied the effect of fat content on thermal inactivation of *E. coli* O157:H7 in beef and poultry. Line et al. (1991) found that the *D*-values of *E. coli* O157:H7 increased from 78.2 to 115.5 min at 51.7°C, from 4.1 to 5.3 min at 57.2°C and from 0.3 to 0.5 min at 62.8°C when the fat content increased in ground beef from 2 to 30.5%. Ahmed et al. (1995) found that when the fat content increased from 3 to 11% in turkey and chicken meat, the *D*-values increased at temperatures of 50–60°C. For chicken, the *D*-value at 50°C increased from 65.24 to 105.5 min, at 55°C the value increased from 8.76 to 9.74 min, and at 60°C it increased from 0.38 to 0.55 min. For turkey, the *D*-value at 50°C increased from 70.41 to 115 min, at 55°C the value increased from 6.37 to 8.76 min, and at 60°C it increased from 0.55 to 0.58 min. Smith et al. (2001) reported that the *E. coli* O157:H7 was more heat stable in beef containing 19% fat than 4.8% fat. Theories behind increased heat resistance of microorganisms in food products with higher fat contents relate to lower heat conductivity or reduced water activity (a_w) in fat portion.

The thermal resistance of foodborne pathogens in food increases with the addition of salt, curing salts, and food additives (Farber, Huges, Holley, & Brown, 1989; Mackey, Pritchet, Norris, & Mead, 1990; Juneja, 2003; Juneja & Eblen, 1999; Murphy, Osaili, Duncan, & Marcy, 2004a). Murphy et al (2004a) found that the *D*-value of *L. monocytogenes* in ground chicken thigh and leg meat with the addition of 4.8% sodium lactate was 53–75% higher than that in the meat without sodium lactate, but, the thermal resistance of a cocktail of *Salmonella* in meat was not affected with addition of sodium lactate. Juneja (2003) found that sodium lactate and sodium diacetate increased thermal resistance of *L. monocytogenes* in meat. The *D*-values of meat mixed with both 4.8% sodium lactate and 0.25% sodium diacetate at 60°C, 65°C, 71.1°C, and 73.9°C increased from 4.67, 0.75, 0.17, and 0.04 min to 13.95, 1.81, 0.16, and 0.07 min, respectively. The *D*-values of *L. monocytogenes* in meat treated with 4.8% sodium lactate alone increased to 14.34, 2.23, 0.22, and 0.13 min at the relevant temperatures. The *D*-values of meat mixed with 0.25% sodium diacetate increased to 4.69, 0.85, 0.17, and 0.08 min at the relevant temperatures. Huang and Juneja (2003) reported that sodium lactate (up to 4.5%) had

no effect on thermal inactivation at 55–65°C of *E. coli* O157:H7 in ground beef. Maurer et al. (2000) and Juneja and Eblen (1999) found that NaCl increased the thermal resistance of *Salmonella* in ground turkey and *L. monocytogenes* in beef gravy, respectively. The science behind decreased heat resistance of microorganisms in food products with addition of salts relates to decreases in water activity, a_w. As the a_w of the heated medium decreased, the thermal resistance of the cells increased (Carlson, Marks, Booren, Ryser, & Orta-Ramirez, 2005). Carlson et al. (2005) found that the thermal resistance of *Salmonella* decreased 64% when decreasing meat a_w from 0.99 to 0.95. Juneja (2003) and Juneja and Eblen (1999) found that a reduction in a_w of ground meat decreased the penetration of the heat through the food, thus increasing the resistance of the pathogens to heat.

Pathogen's Characteristics

The pathogen's characteristics include the type and the strain of the bacterial pathogen, its growth condition, and the exposure to stresses.

In general, Gram-positive bacteria are more heat resistance than Gram-negative bacteria. The temperature at which cultures were grown would have an effect on the thermal resistance of the bacteria. Cultures grown at higher temperatures have been shown to be more thermally resistant than those grown at lower temperatures (Juneja & Eblen, 1999). Furthermore, length of incubation has an effect on the thermal resistance of the bacteria; cells in the stationary phase of growth are generally more heat resistant than cells in the logarithmic phase of growth (Lou & Yousef, 1996). Smith et al. (2001) reported that a *Salmonella* cocktail in ground beef was more heat resistant during stationary growth phase than during logarithmic growth phase. The *D*-values at temperature 55–63°C were in the range of 18.66–0.20 min and 16.34–0.15 min for the pathogen in stationary and log phase, respectively.

Farber and Brown (1990) reported that *L. monocytogenes* that had been previously heat shocked at 48°C for 120 min showed a 2.4-fold increase in the *D*-value at 64°C. Wesche, Marks, and Ryser (2005) found that heat shock at 54°C for 30 min increased the thermal resistance of *Salmonella* in comminuted turkey. While cold shock at 4°C for 2 h decreased the thermal resistance of the cells. Similarly, Mackey and Derrick (1987) observed that heat shock at 48°C for 30 min increased the thermal resistance of *Salmonella* Thompson at 54°C and 60°C about 2.4- and 2.7-fold, respectively, in minced beef. Juneja, Klein, and Marmer (1998) reported that thermal resistance of *E. coli* O157:H7 in beef gravy and ground beef increased about 1.5-fold when the cells were pre-exposed to sub-lethal heat. Smith et al. (2001) reported that starvation of *Salmonella* in peptone water for 14 days at 4°C decreased the thermal resistance of the microbe at 55–63°C in ground beef. Miller, Bayles, and Eblen (2000) reported a reduction in the *D*-value at 60°C of *L. monocytogenes* inoculated onto the surface of frankfurter skin following cold shocking at 0°C for 3 h.

Thermal Inactivation of Foodborne Pathogens in Meat and Poultry Products

Thermal Inactivation of *Salmonella* in Meat

Thermal inactivation kinetics of a cocktail of *Salmonella* in beef has been reported. Goodfellow and Brown (1978) determined the *D*-values for a cocktail of *Salmonella* at temperatures of 51.6–62.7°C. The *D*-values ranged from 62 to 0.6 min, and the *z*-value was 5.6°C. Orta-Ramirez et al. (1997) reported *D*-values of *Salmonella* Senftenberg in ground beef at 58°C and 64°C of 15.17 and 2.08 min, respectively, and *z*-value of 6.25°C. Juneja, Eblen, & Ransom (2001) found that the *D*-values of a cocktail of *Salmonella* in beef at temperatures of 58–65°C ranged from 8.65 to 0.67 min, and the *z*-value was 6.01°C while in ground pork the values ranged from 6.68 to 0.87 min and the *z*-value was 7.10°C. Juneja and Eblen (2000) reported *D*-values at 58–65°C for eight strain cocktail of *Salmonella* Typhimurium DT104 in beef containing 7–24% fat. The *D*-values in beef containing 7%, 12%, 18%, and 24% fat ranged from 3.22 to 0.70 min, 2.46 to 0.34 min, 2.49 to 0.41 min, and 1.61 to 0.18 min, respectively. Smith et al. (2001) found that that the *D*-values of a cocktail of *Salmonella* at stationary phase at 55–63°C in high-fat (19.1%) ground beef ranged from 18.66 to 0.20 min, and the *z*-value was 4.08°C. Quintavalla, Larini, Mutti, and Barbuti (2001) compared among the thermal resistance of eight *Salmonella* strains (*Salmonella* Typhimurium strains (ATCC 14028, I33 and I116), *Salmonella* Derby B4373, *Salmonella* Potsdam I133, *Salmonella* Menston I79, *Salmonella* Eppendorf I66, and *Salmonella* Kingston I124) in pork meat containing curing additives. They found that *Salmonella* Potsdam strain was the most resistant one, with *D*-values at 58–63°C ranged from 4.8 to 0.3 min, while the most sensitive strain was *Salmonella* Kingston, with *D*-values ranged from 2.79 to 0.24 min, at the same temperatures. Murphy, Duncan, Johnson, Davis, and Smith (2002) determined *D*- and *z*-values at temperatures of 55–70°C for a cocktail of *Salmonella* in different commercial meat products including beef patties and blended beef and turkey patties. The *D*-values in beef patties and blended beef and turkey patties ranged from 9.09 to 0.25 min and from 20.58 to 0.37 min, respectively, and the *z*-values were 9.14 and 8.35°C, for beef patties and blended beef and turkey patties, respectively. Murphy, Beard, Martin, Keener, and Osaili (2004) reported *D*-values for a cocktail of *Salmonella* in the range of 41.02–0.10 min in ground and formulated beef/turkey at 55–70°C. Murphy, Beard, Martin, Duncan, et al. (2004) and Murphy, Martin, Duncan, Beard, and Marcy (2004) reported *D*-values at 55–70°C for a cocktail of *Salmonella* in ground pork and ground beef in the range of 45.87–0.083 min and 37.05–0.066 min, respectively, and *z*-values of 5.89°C and 5.74°C, respectively. Osaili et al. (2007) and Osaili, Griffis, Martin, Beard, et al. (2006) reported that the *D*-values of a cocktail of *Salmonella* in chicken fried beef and breaded pork patties at 55–70°C ranged from 67.68 to 0.22 min and 69.48 to 0.29 min and the *z*-value of 6.0°C and 6.2°C, respectively.

The differences in the thermal inactivation parameters (*D*- and *z*-values) could be due to differences in methodology used for recovery of survivors, physiological condition of the cells sample size, or meat composition. Bigger samples might have longer come-up time leading to larger *D*-values.

Table 9.1 shows some of the reported *D*- and *z*-values for *Salmonella* in meat.

Thermal Inactivation of *Salmonella* in Poultry

Thermal inactivation kinetics of *Salmonella* in chicken breast and turkey meat has been studied. Hussemann and Buyske (1954) studied the thermal death temperature–time relationships of *Salmonella* Typhimurium in chicken muscle. *Salmonella* was mixed with ground chicken muscle and heated in glass test tubes in an oil bath to temperatures of 75°C, 80°C, 85°C, and 90°C for 3, 5, 10, 15, 20, and 40 min. At 75°C for 5 min, 75% of the samples showed survivors of *Salmonella*. At 90°C for 15, 20, and 40 min, there were no survivors of *Salmonella*. Veeramuthu, Price, Davis, Booren, and Smith (1998) calculated the *D*-values of *Salmonella* Senftenberg in turkey thigh (4.35% fat) at temperatures of 55, 60, and 65°C. The values were 211.4–227.2 min, 13.2–13.6 min and 3.11–3.4 min, respectively, depending on the recovery method used, and *z*-values were 5.4–5.6°C. At temperatures of 56–63°C, Mazzotta (2000) found that the *D*-values of a cocktail of *Salmonella* in chicken breast meat ranged from 3.2 to 0.18 min and the *z*-value was 5.7°C. Juneja, Eblen, & Ransom (2001) found that the *D*-values of a cocktail of *Salmonella* at temperatures of 58–65°C ranged from 7.42 to 0.80 min in ground turkey (9% fat), and from 7.08 to 0.59 min in ground chicken (7% fat). The *z*-values in turkey and chicken were 6.88 and 6.11°C, respectively. At temperatures of 55–70°C, Murphy, Marks, Johnson, and Johnson (2000) and Murphy, Duncan, Berrang, Marcy, and Wolfe (2002) found the *D*-values for a cocktail of *Salmonella* in raw and fully cooked chicken breast meat were 30.10–0.24 min, 24.071–0.097 min, respectively, and the *z*-value was 6.53 and 6.26°C, respectively. Murphy, Duncan, Beard, and Driscoll (2003) compared among the thermal resistance of a cocktail of *Salmonella* in fully cooked poultry products including duck muscle meat, duck skin, and turkey breast meat. The *D*-values at 55–70°C in the relevant products ranged from 28.57 to 0.11 min, 25.32 to 0.17 min, 24.69 to 0.12 min, and 24.07 to 0.10 min, respectively. Murphy et al. (2000) and Murphy, Duncan, Johnson, et al. (2002) determined *D*- and *z*-values at temperatures of 55–70°C for a cocktail of *Salmonella* in ground chicken breast meat and in different commercial meat products including chicken patties and chicken tender. The *D*-values in the ground chicken breast, chicken patties, and chicken tender ranged from 30.1 to 0.238 min, 26.97 to 0.32 min, and 22.37 to 0.32 min, respectively, and the *z*-values were 6.53°C, 7.60°C, and 7.61°C for ground chicken breast, chicken patties, and chicken tender, respectively. Murphy, Martin, et al. (2004) reported *D*-values at 55–70°C for a cocktail of *Salmonella* in ground turkey in the range of 43.10–0.096 min and *z*-value of 5.96°C. Murphy et al. (2004a) and Murphy, Osaili, Duncan, and Marcy (2004b) studied

Table 9.1 *D*- and *z*-values of *Salmonella* spp in meat

Medium	Strain	Temperature °C											*z*-value	References
		55.0	57.5	58.0	60.0	61.0	62.5	63.0	64.0	65.0	67.5	70.0		
Beef	Cocktail	37.05	18.35		6.90		2.62			1.03	0.30	0.07	5.74	Murphy, Martin, et al. (2004)
Beef (12% fat)	Cocktail			2.46	1.43		0.24			0.34			5.66	Juneja and Eblen (2000)
Beef (12.5% fat)	Cocktail			8.65	5.48		1.50			0.67			6.01	Juneja, Eblen, and Ransom (2001)
Beef (18% fat)	Cocktail			2.49	0.48		1.98			0.41			5.47	Juneja and Eblen (2000)
Beef (19.1% fat)	Senftenberg			21.79		3.38			0.92				4.51	Smith et al. (2001)
Beef (19.1% fat)	DT104-10127	21.98		2.63		0.65			0.16				4.28	Smith et al. (2001)
Beef (24% fat)	Cocktail			1.61	0.48		2.35			0.18			6.99	Juneja and Eblen (2000)
Beef (4.8% fat)	DT104-01071	10.27		2.06		0.43			0.14				4.77	Smith et al. (2001)
Beef (7% fat)	Typhimurium			3.22	1.75		0.27			0.70			8.08	Juneja and Eblen (2000)
Beef patties (19% fat)	Cocktail	9.09	7.70		4.80		2.40			0.97	0.57	0.25	9.14	Murphy, Duncan, Johnson, et al. (2002)
Beef (4.8% fat)	DT104-10127	9.05		2.26		0.57		0.15	0.15				5.07	Smith et al. (2001)
Beef (4.8% fat)	DT104-10601	10.55		2.15		0.41			0.07				4.13	Smith et al. (2001)
Beef/turkey (10% fat)	Cocktail	41.02	15.15		5.60		2.07			0.76	0.28	0.10	5.83	Murphy, Beard, Martin, Keener, et al. (2004)
Beef/turkey (20% fat)	Cocktail	20.58	12.89		4.42		2.04			1.03	0.71	0.37	8.35	Murphy, Duncan, Johnson, et al. (2002)
Franks (24% fat)	Cocktail	14.73	6.67		5.63		4.38			1.64	0.78	0.39	9.83	Murphy, Duncan, Johnson, et al. (2002)
Beef (14.6% fat)	Cocktail	67.68	27.31		15.51		5.61			2.00	0.50	0.22	6.00	Osaili, Griffis, Martin, Beard, et al. (2006)
Beef	Senftenbery			15.17				2.08					6.25	Ort-Ramirez et al. (1997)
Beef (19.1% fat)	Cocktail	18.66		3.39		0.57		0.20					4.08	Smith et al. (2001)
Beef (25% fat)	Cocktail	19.31	9.30		4.72								8.06	Juneja (2003)
Pork	Cocktail	45.87	26.67		5.07		2.56			1.91	0.36	0.08	5.89	Murphy, Beard, Martin, Duncan, et al. (2004)
Pork	Cocktail	69.48	29.99		15.20		7.71			2.64	0.61	0.29	6.20	Osaili et al. (2007)
Pork (8.5% fat)	Cocktail			6.68	6.65		1.62			0.87			7.10	Juneja, Eblen, and Ransom (2001)

the thermal inactivation of a cocktail of *Salmonella* in ground chicken thigh/leg meat alone or mixed with 4.8% sodium lactate and in skin as varieties of value-added poultry products contain skin. They reported *D*-values of 43.76–0.07 min in the plain meat, 43.78–0.08 min in meat mixed with 4.8% sodium lactate, and 43.33–0.09 min in chicken skin at 55–70°C. The *z*-values were 5.46°C, 5.46°C, and 5.56°C in the relevant products. The *D*-values of *Salmonella* in meat were significantly different than that in skin at 60–70°C.

Table 9.2 shows some of the reported *D*- and *z*-values for *Salmonella* in poultry.

Thermal Inactivation of *L. monocytogenes* in Meat

Thermal inactivation kinetics of *L. monocytogenes* in beef meat has been calculated. Farber et al. (1989) determined the thermal resistance of a cocktail of *L. monocytogenes* at 56–64°C in pork–beef meat mixture (66% pork and 33% beef) and pork–beef mixture with cure. The *D*-values in plain meat at 56–62°C ranged from 14.18 to 1.01 min and the values in meat with cure at 58–64°C ranged from 50.0 to 1.28 min. Boyle, Sofos, and Schmidt (1990) found that *D*-values of *L. monocytogenes* Scott A in meat slurry at temperatures of 60–70°C were 2.54–0.23 min. Fain et al. (1991) found that the *D*-values for *L. monocytogenes* Scott A in lean beef at temperatures of 51.2–62.7°C were 81.3–0.6 min, respectively, and the value was 9.3̊F. Schoeni, Brunner, and Doyle (1991) determined the thermal resistance of a cocktail of *L. monocytogenes* in ground beef roast and fermented beaker sausage. The *D*-values in ground beef roast at 54.4–62.8°C were in the range of 22.4–2.56 min and in fermented beaker sausage at 48.9–60°C were in the range of 98.6–9.13 min. Doherty et al. (1998) calculated the *D*-values for *L. monocytogenes* in minced beef heated in vacuum bags at temperatures of 50–60°C. The values ranged from 43.5 to 0.24 min. Murphy, Duncan, Johnson, et al. (2002) determined *D*- and *z*-values at temperatures of 55–70°C for *L. innocua*, which was developed as a heat resistance model for *L. monocytogenes,* in different commercial meat products including beef patties and blended beef and turkey patties. The *D*-values in beef patties and blended beef and turkey patties ranged from 19.52 to 0.29 min and from 46.08 to 0.18 min, respectively, and the *z*-values were 8.67 and 6.41°C, for beef patties and blended beef and turkey patties, respectively. Murphy, Beard, Martin, Duncan, et al. (2004) and Murphy, Martin, et al. (2004) reported *D*-values at 55–70°C for a cocktail of *L. monocytogenes* in ground pork and ground beef in the range of 47.17–0.085 min and from 36.91 to 0.063 min, respectively, and *z*-values of 5.92°C and 6.01°C, respectively. Murphy, Beard, Martin, Keener, et al. (2004) reported *D*-values for a cocktail of *L. monocytogenes* in the range of 50.35–0.13 min in ground and formulated beef/turkey at 55–70°C. Osaili, Griffis, Martin, Beard, et al. (2006) and Osaili et al. (2007) reported *D*-values in chicken fried beef and breaded pork patties at 55–70°C of 81.37–0.31 min and from 150.46 to 0.43 min and *z*-value of 6.1°C and 5.9°C, respectively.

Table 9.2 *D*- and *z*-values of *Salmonella* spp in poultry

Medium	Strain	Temperature °C											*z*-value	References
		55.0	56.0	57.5	58.0	60.0	62.0	62.5	63.0	65.0	67.5	70.0		
Chicken			3.20			0.60	0.31		0.18				5.70	Mazzotta (2000)
Chicken	Cocktail	30.10		12.90		5.88		2.51		1.16	0.36	0.24	6.53	Murphy et al. (2000)
Chicken (meat)	Cocktail	43.76		13.66		5.72		1.62		0.55	0.19	0.07	5.34	Murphy, Osaili, Duncan, and Marcy (2004b)
Chicken (skin)	Cocktail	43.33		14.09		7.31		2.21		0.79	0.23	0.09	5.56	Murphy, Osaili, Duncan, and Marcy (2004b)
Chicken breast	Cocktail										0.29	0.18		Murphy et al. (1999)
Chicken thigh/leg meat	Cocktail	43.76		13.66		5.72		1.62		0.55	0.19	0.07	5.46	Murphy, Osaili, Duncan, and Marcy (2004a)
Chicken thigh/leg meat	Cocktail	43.78		13.00		4.76		1.77		0.66	0.20	0.08	5.46	Murphy, Osaili, Duncan, and Marcy (2004a)
Chicken (7% fat)	Cocktail				7.08	5.20		1.36		0.59			6.11	Juneja, Eblen, and Ransom (2001)
Cooked turkey breast	Cocktail	24.69		8.67		5.20		1.85		0.62	0.19	0.12	6.23	Murphy, Duncan, Beard, et al. (2003)
Cooked chicken	Cocktail	24.07		9.60		3.83		1.53		0.61	0.24	0.10	6.26	Murphy, Beard, Martin, Duncan, et al. (2004)
Cooked chicken breast	Cocktail	24.07		9.60		3.83		1.53		0.61	0.24	0.10	6.26	Murphy, Duncan, Beard, et al. (2003)
Chicken (12% fat)	Cocktail				9.04	5.50		1.30		0.50				Juneja, Eblen, and Marks (2001)
Chicken (2% fat)	Cocktail				7.38	4.83		1.14		0.41				Juneja, Eblen, and Marks (2001)
Chicken (6.3% fat)	Cocktail				7.33	4.68		1.16		0.51				Juneja, Eblen, and Marks (2001)
Chicken (9% fat)	Cocktail				8.54	5.40		1.16		0.53				Juneja, Eblen, and Marks (2001)
Turkey (1% fat)	Cocktail				7.50	4.56		1.53		0.59				Juneja, Eblen, and Marks (2001)
Turkey (10% fat)	Cocktail				6.91	5.13		1.45		0.57				Juneja, Eblen, and Marks (2001)
Turkey (12% fat)	Cocktail				7.41	5.43		1.78		0.59				Juneja, Eblen, and Marks (2001)
Turkey (7% fat)	Cocktail				7.71	4.94		1.85		0.55				Juneja, Eblen, and Marks (2001)
Raw chicken patties	Cocktail	26.97		14.55		8.09		3.98		1.39	0.61	0.32	7.60	Murphy, Duncan, Johnson, et al. (2002)
Raw chicken tenders	Cocktail	22.37		9.92		8.50		4.55		1.25	0.38	0.32	7.61	Murphy, Duncan, Johnson, et al. (2002)
Roast duck skin	Cocktail	25.32		14.47		4.30		2.46		1.21	0.49	0.17	7.01	Murphy, Duncan, Beard, et al. (2003)
Roast duck meat	Cocktail	28.57		14.31		6.79		2.09		0.58	0.20	0.11	5.82	Murphy, Duncan, Beard, et al. (2003)
Turkey (5.4% fat)	Cocktail	43.10		10.69		6.73		2.54		1.51	0.29	0.10	5.96	Murphy, Martin, et al. (2004)
Turkey (9% fat)	Cocktail				7.42	4.82		1.51		0.80			6.88	Juneja, Eblen, and Ransom (2001)

Table 9.3 shows some of the reported *D*- and *z*-values for *L. monocytogenes* in meat.

Thermal Inactivation of *L. monocytogenes* in Poultry

Murphy et al. (2000) and Murphy, Duncan, Berrang, et al. (2002) studied the thermal inactivation of *L. innocua* in raw and fully cooked chicken breast meat at temperatures of 55–70°C. The *D*-values ranged from 50.8 to 0.187 min and 56.169 to 0.126 min, respectively, and the *z*-values were 6.29 and 5.67 °C, respectively. Murphy, Duncan, Johnson, et al. (2002) determined the *D*- and *z*-values for *L. innocua* in different commercial meat products including chicken patties and chicken tenders at temperatures of 55–70°C. The *D*-values of chicken patties and chicken tenders ranged from 191.94 to 0.21 min and 128.21 to 0.29 min, respectively, and the *z*-values were 4.86°C and 5.55°C for chicken patties and chicken tenders, respectively. Murphy, Duncan, Beard, et al. (2003) compared among the thermal resistance of a cocktail of *L. monocytogenes* in fully cooked poultry products including duck muscle meat, duck skin, turkey breast meat, and chicken breast meat. The *D*-values at 55–70°C in the relevant products ranged from 131.58 to 0.11 min, 82.65 to 0.21 min, 119.05 to 0.21 min, and 51.02 to 0.13 min, respectively. Murphy, Martin, et al. (2004) reported *D*-values at 55–70°C for a cocktail of *L. monocytogenes* in ground turkey in the range of 33.11–0.12 min and *z*-value of 5.90°C. Murphy et al. (2004a, 2004b) studied the thermal inactivation of a cocktail of *L. monocytogenes* in ground chicken thigh/leg meat alone or with 4.8% sodium lactate and in skin and reported *D*-values at 55–70°C of 38.94–0.04 min in the plain meat, 82.75–0.11 min in the meat with 4.8% sodium lactate and 34.05–0.05 min in the skin. The *z*-values were 5.08°C, 5.28°C, and 5.27°C in the relevant products. The *D*-values of *L. monocytogenes* in meat were significantly different than those in skin at a temperature of 60–70°C.

Table 9.4 shows some of the reported *D*- and *z*-values for *L. monocytogenes* in poultry.

Thermal Inactivation of *E. coli* O157:H7 in Meat

Line et al. (1991) determined the *D*- and *z*-values of *E. coli* O157:H7 in the lean and fatty ground beef using plate count agar. They reported *D*-values at 51.7–62.7°C in the range of 78.2–0.30 min and 115.5–0.47 min in lean and fatty meat, respectively and *z*-values of 8.3 and 8.4°F in lean and fatty meat, respectively. Ahmed et al. (1995) reported *D*-values for *E. coli* O157:H7 isolate 204P in the range of 55.34–0.45, 80.66–0.46 min and 92.67–0.47 min in beef containing 7%, 10%, and 20% fat, respectively, and *D*-values in the range of 49.50–0.37, 62.90–0.46, and 80.64–0.55 in pork sausage containing 7%, 10%, and 20% fat, respectively, at temperature range of 50–60°C. Also, they reported *z*-values of 4.78°C, 4.44°C, and 4.35°C for the pathogen in beef containing 7%, 10%, and 20% fat, respectively, and 4.72, 4.67, and 4.61 in pork sausage

Table 9.3 *D*- and *z*-values of *Listeria monocytogenes* in meat

Medium	Strain	Temperature °C												*z*-value	References
		55	57.5	58	60	62	62.5	64	65	66	67.5	68	70		
Beef	Cocktail	21			3.8				0.93					7.2	Mackey et al. (1990)
Beef	Cocktail	36.91	11.28		3.91		2.4		1.41		0.39		0.063	6.01	Murphy, Martin, et al. (2004)
Beef (14.6%)	Cocktail	81.32	40.49		22.98		7.15		2.81		0.93		0.31	6.1	Osaili, Griffis, Martin, Beard, et al. (2006)
Beef (30.5% fat)	Scott A						1.2							11.4	Fain et al. (1991)
Beef (2% fat)	Scott A						0.6							9.3	Fain et al. (1991)
Beef (<7% fat)	Cocktail				8.32	4.2		2.19		0.94		0.35		5.98	Gaze, Brown, Gaskell, and Banks (1989)
Beef (<7% fat)	Cocktail				6.27	2.9		2.21		0.93		0.27		5.98	Gaze et al. (1989)
Beef/turkey	Cocktail	50.35	18.6		6.87		2.54		0.94		0.35		0.13	5.9	Murphy, Beard, Martin, Keener, et al. (2004)
Beef roast	Cocktail				4.47										Schoeni et al. (1991)
Beef (25% fat)	Cocktail	27.69	9.63		4.18									6.1	Juneja (2003)
Ground meat	Cocktail			6.39	3.12	1.01								4.92	Farber et al. (1989)
Meat + cure	Cocktail			50	16.7	7.06		1.28						3.5	Farber et al. (1989)
Hot dog batter	Cocktail			14.6		3.2				0.65				5.9	Mazzotta and Gombas (2001)
	Cocktail			15.5		3.3				0.7				6	Mazzotta and Gombas (2001)
Hot dog batter	Cocktail			11		1.8				0.4				5.6	Mazzotta and Gombas (2001)
Hot dog batter	Cocktail			8.1		1.8				0.23				5.2	Mazzotta and Gombas (2001)
Meat slurry (1.23%)	Scott A				2.54				0.75				0.23		Boyle et al. (1990)
Minced beef	Cocktail	3.4			0.31									4.9	Bolton et al. (2000)
Minced beef	Cocktail	3.2			0.15									4.2	Bolton et al. (2000)
Minced beef	Cocktail													5.9	Bolton et al. (2000)
Beef	Cocktail				1.62										Schoeni et al. (1991)
Pork (40.2% fat)	Cocktail	47.17	22.32		5.61		2.87		1.5		0.44		0.085	5.92	Murphy, Beard, Martin, Duncan, et al. (2004)
Pork (23% fat)	Cocktail	150.46	55.08		20.61		10.64		3.08		1.15		0.43	5.9	Osaili et al. (2007)
Sausage	Cocktail				9.13										Schoeni et al. (1991)

Table 9.4 *D*- and *z*-values of *Listeria monocytogenes* in poultry

Medium	Strain	Temperature °C											*z*-value	References
		55	57.5	60	62	62.5	64	65	66	67.5	68	70		
Chicken (thigh/leg)	Cocktail	38.94	9.67	2.04		1.01		0.30		0.10		0.04	5.08	Murphy, Osaili, Duncan, and Marcy (2004b)
Chicken (47.4% fat, skin)	Cocktail	34.05	10.19	3.95		1.37		0.41		0.13		0.05	5.27	Murphy, Osaili, Duncan, and Marcy (2004b)
Chicken breast	Cocktail			5.29	2.51		1.56		0.68		0.38	0.16	6.72	Gaze et al. (1989)
Chicken breast	Cocktail			5.02	2.21		1.84		0.95		0.41	0.20	7.39	Gaze et al. (1989)
Chicken breast	Cocktail	13.00		8.70				0.52				0.13	6.30	Mackey et al. (1990)
Chicken leg	Cocktail	14.00		5.60				0.53				0.11	6.70	Mackey et al. (1990)
Chicken leg quarter meat	Cocktail	131.58	23.64	9.84		2.23		0.93		0.42		0.11	5.76	Murphy, Driscoll, et al. (2003)
Chicken leg quarter skin	Cocktail	82.65	21.69	8.90		3.57		1.16		0.40		0.21	5.04	Murphy, Driscoll, et al. (2003)
Cooked chicken breast	Cocktail	51.02	13.93	8.49		2.60		0.65		0.29		0.13	5.71	Murphy, Duncan, Beard, et al. (2003)
Cooked turkey breast	Cocktail	119.05	40.16	16.70		4.54		1.29		0.46		0.21	5.29	Murphy, Duncan, Beard, et al. (2003)
Duck muscle	Cocktail	131.58	23.64	9.84		2.23		0.93		0.42		0.11	5.04	Murphy, Duncan, Beard, et al. (2003)
Duck skin	Cocktail	82.65	21.69	8.90		3.57		1.16		0.40		0.21	5.76	Murphy, Duncan, Beard, et al. (2003)
Thigh and leg meat	Cocktail	82.75	26.54	7.78		2.50		1.07		0.40		0.11	5.28	Murphy, Osaili, Duncan, and Marcy (2004a)
Thigh and leg meat	Cocktail	38.94	9.67	2.04		1.01		0.30		0.10		0.04	5.28	Murphy, Osaili, Duncan, and Marcy (2004a)
Turkey	Cocktail	33.11	17.09	8.05		2.88		0.92		0.28		0.12	5.90	Murphy, Martin, et al. (2004)

containing 7%, 10%, and 20% fat, respectively. Juneja, Snyder, and Marmer (1997) reported *D*-values in the range of 21.13–0.39 min at 55–65°C in beef and *z*-value of 5.98°C. Smith et al. (2001) reported *D*-values in low-fat beef (4.8%) in the range of 20.08–0.16 min and in high-fat beef (19.1%) in the range of 22.47–0.18 min at 55–63°C. The *z*-values in high- and low-fat beef were 3.79°C and 3.60°C, respectively. Byrne, Bolton, Sheridan, Blair, and McDowell (2002) found that commercial production and product formulation had an effect on the heat resistance of *E. coli* O157:H7 (NCTC 12900) in beef burger. They found that the *D*-values at 55–65°C in quality formulations subjected to freezing ranged from 9.3 to 0.5 min compared with 20.8 to 0.6 min in unfrozen sample. While the *D*-values in unfrozen economy formulation samples ranged from 41.1 to 0.7 min and in frozen samples ranged from 11.7 to 0.6 min at 55°C, 60°C, and 65°C, respectively. Huage and Juneja (2003) reported *D*-values in lean ground beef containing 0, 1.5, 3.0, and 4.5 sodium lactate in the range of 11.13–0.75, 11.16–0.73, 10.91–0.71, and 11.02–0.73, respectively, at 55–65°C. Murphy, Beard, Martin, Duncan, et al. (2004) and Murphy, Martin, et al. (2004) and Murphy, Davidson, and Marcy (2004) reported *D*-values for a cocktail of *E. coli* O157:H7 in ground pork, raw formulated frank (containing beef, pork, and chicken), fully cooked frank, and ground beef in the range of 33.44–0.048 min, 21.36–0.031 min, 24.91–0.038 min, and 21.56–0.055 min, respectively, at 55–70°C and *z*-values of 4.94°C, 5.07°C, 5.08°C, and 5.43°C, respectively. Murphy, Beard, Martin, Keener, et al. (2004) reported *D*-values for a cocktail of *E. coli* O157:H7 in the range of 23.23–0.03 min in ground and formulated beef/turkey at 55–70°C. Osaili, Griffis, Martin, Beard, et al. (2006) and Osaili et al. (2007) reported *D*-values in chicken fried beef and breaded pork patties at 55–70°C of 27.62–0.04 min and 32.11–0.08 min and *z*-values of 5.2°C and 5.4°C, respectively.

Table 9.5 shows some of the reported *D*- and *z*-values for *E. coli* O157:H7 in meat.

Thermal Inactivation of *E. coli* O157:H7 in Poultry

Ahmed et al. (1995) reported *D*-values for *E. coli* O157:H7 isolate 204P in the range of 65.24–0.38 and 105.5–0.55 min in chicken containing 3% and 11% fat, respectively, and *D*-values in the range of 70.41–0.55 and 115.0–0.58 min in turkey containing 3% and 11% fat, respectively, at temperature range of 50–60°C. They reported *z*-value of 4.48°C and 4.38°C for the pathogen in chicken containing 3 and 11% fat, respectively, and 4.74 and 4.35 in turkey containing 3 and 11% fat, respectively. Kotrola and Conner (1997) reported *D*-values at 52–60°C in low- (3%) and high-fat (11%) content turkey meat with or without 8% NaCl, 4% sodium lactate or a mixture of 8% NaCl, 4% sodium lactate and 0.5% polyphosphate. The *D*-values ranged from 42.3 to 0.9 min, 83.6 to 1.4 min, 75.1 to 0.9 min, and –103.3 to 2.4 min in plain low-fat meat, meat with NaCl, meat with sodium lactate, and meat with salt mix, respectively. In plain high-fat meat, meat with NaCl, meat with sodium lactate, and meat

Table 9.5 *D*- and *z*-values of *Escherichia coli* O157:H7 in meat

Medium	Strain	Temperature °C												*z*-value	References
		50	52	55	57.5	58	60	61	62.5	63	65	67.5	70		
Beef	Cocktail			21.56	8.14		1.96		0.88		0.32	0.12	0.06	5.43	Murphy, Martin, et al. (2004)
Beef	Cocktail			11.13	2.64		1.71		1.03		0.75				Huang and Juneja (2003)
Beef (10% fat)	204P	80.66		15.3			0.46							4.44	Ahmed et al. (1995)
Beef (14.6% fat)	Cocktail			27.62	11.35		2.5		0.75		0.23	0.11	0.04	5.2	Osaili, Griffis, Martin, Beard, et al. (2006)
Beef (19.1% fat)	204P			22.47		2.05		0.32		0.18				3.6	Smith et al. (2001)
Beef (2% fat)	NR		78.2							0.3				8.3	Line et al. (1991)
Beef (20% fat)	204P	92.67		19.26			0.47							4.35	Ahmed et al. (1995)
Beef (25% fat)	Cocktail			20.89	7.77		3.39							6.33	Juneja (2003)
Beef (3% Na-lactate)	Cocktail			10.91	2.55		1.65		0.97		0.71				Huang and Juneja (2003)
Beef (30.5% fat)	NR		115.5							0.47				8.4	Line et al. (1991)
Beef (4.5% Na-lactate)	Cocktail			11.02	2.61		1.72		1.03		0.73				Huang and Juneja (2003)
Beef (4.8% fat)	204P			20.08		1.22		0.32		0.16				3.79	Smith et al. (2001)
Beef (7% fat)	204P	55.34		11.4			0.45							4.78	Ahmed et al. (1995)
Beef (10% fat)	Cocktail			21.13	4.95		3.17		0.93		0.39				Juneja et al. (1997)
Beef (1.5% Na-lactate)	Cocktail			11.16	2.71		1.69		1.01		0.73				Huang and Juneja (2003)
Beef/turkey (10% fat)	Cocktail			23.23	7.43		2.38		0.76		0.24	0.08	0.03	5.15	Murphy, Beard, Martin, Keener, et al. (2004)
Cooked franks (27.2% fat)	Cocktail			24.91	11.9		3.48		1		0.24	0.1	0.04	5.08	Murphy, Davidson, et al. (2004)
Economy formulation	NCTC 12900			41.1			4.2				0.7				Byrne et al. (2002)
Pork (10% fat)	204P	62.9		7.83			0.46							4.72	Ahmed et al. (1995)
Pork (23% fat)	Cocktail			33.44	10.37		3.22		0.8		0.26	0.08	0.05	4.94	Murphy, Beard, Martin, Duncan, et al. (2004)
Pork (23% fat)	Cocktail			32.11	15.38		3.02		1.07		0.37	0.13	0.08	5.4	Osaili et al. (2007)
Pork (30% fat)	204P	80.64		11.28			0.55							4.61	Ahmed et al. (1995)
Pork (7% fat)	204P	49.5		6.37			0.37							4.62	Ahmed et al. (1995)
Quality formulation	NCTC 12900			20.8			2.7				0.6				Byrne et al. (2002)
Raw franks (17.2% fat)	Cocktail			21.36	6.54		1.83		0.66		0.15	0.05	0.03	5.07	Murphy, Davidson, et al. (2004)

NR: not reported.

with salt mix the *D*-values ranged from 38.5 to 0.9 min, 77.9 to 1.6 min, 74.6 to 1.0 min and –95.2 to 2.7 min, respectively. The *z*-values were 4.78°C, 4.30°C, 4.60°C, and 5.08°C in plain low-fat meat, meat with NaCl, meat with sodium lactate, and meat with salt mix, respectively. The *z*-values in plain high-fat meat, meat with NaCl, meat with sodium lactate, and meat with salt mix were 4.45°C, 4.95°C, 4.55°C, and 4.58°C, respectively. Juneja et al. (1997) reported *D*-values for a cocktail of the microorganism in the range of 11.83–0.36 min at 55–65°C in chicken and *z*-value of 6.79°C. Murphy, Martin, et al. (2004) reported *D*-values for a cocktail of *E. coli* O157:H7 in ground turkey in the range of 19.05–0.038 min, respectively, at 55–70°C and *z*-value 5.17°C.

Table 9.6 shows some of the reported *D*- and *z*-values for *E. coli* O157:H7 in poultry.

Control of Foodborne Pathogens in RTE Meat and Poultry Products

Air Impingement Oven Cooking

Air impingement cooking which may be combined with steam is extensively used in the processing of RTE meat and poultry products (Murphy, Duncan, Johnson, & Davis, 2001). In these types of ovens process conditions such as temperature, air velocity, and conveyer belt speed greatly influence the process lethality of foodborne pathogens in meat and poultry products during thermal processing.

Murphy, Johnson, Marks, Johnson, and Marcy (2001) found that cooking of ground chicken breast patties to a final temperature of 70–80°C caused 1–4 *D* reduction in *Salmonella* Senftenberg and 3–5 *D* reduction in *L. innocua* during cooking of the meat in an air convection oven. Murphy, Duncan, Johnson, and Davis (2001) evaluated the thermal inactivation of *Salmonella* spp and *L. innocua* in chicken patties cooked in an air-steam convection oven at an air temperature of 149°C, air velocity of 7–13 m^3/min and a wet bulb temperature of 39–98°C. They found that process lethality of *Salmonella* spp and *L. innocua* in cooked chicken patties were strongly correlated to the center temperature of the patties, for instance, increasing product temperature from 70 to 80°C increased the process lethality more than 30-fold. They also found that cooking humidity (wet bulb temperature) and air velocity affected process lethality. The process lethality decreased with increasing wet bulb temperature from 39 to 98°C. Air velocity affected the heat transfer rate and rate of steam condensation on patty surfaces thus affected the process lethality. In order to achieve the required process lethality which is equivalent to 7-*D* reduction of *Salmonella* spp, patties should be cooked to internal temperature in the range of 70–74°C varying with cooking humidity and air velocity. In similar study, Murphy, Johnson, Duncan, et al. (2001) evaluated the thermal inactivation of *Salmonella* spp and *L. innocua* in chicken patties cooked in air convection ovens at an air

Table 9.6 *D*- and *z*-values of *Escherichia coli* O157:H7 in poultry

Medium	Strain	Temperature °C										*z*-value	References
		50	52	55	57	57.5	60	62.5	65	67.5	70		
Chicken (10% fat)	Cocktail			11.83		3.79	1.63	0.82	0.36			6.79	Juneja et al. (1997)
Chicken (11% fat)	204P	105.5		9.74			0.55					4.38	Ahmed et al. (1995)
Chicken (3% fat)	204P	65.24		8.76			0.38					4.48	Ahmed et al. (1995)
Turkey (5% fat)	Cocktail			19.05		8.65	2.06	0.54	0.25	0.048	0.038	5.17	Murphy, Martin, et al. (2004)
Turkey (11% fat)	204P	115		9.69			0.58					4.35	Ahmed et al. (1995)
Turkey (11% fat)	204P		38.5	11	2.4		0.9					4.45	Kotrola and Conner (1997)
Turkey (3% fat)	204P	70.41		6.37			0.55					4.74	Ahmed et al. (1995)
Turkey (3% fat)	204P		42.3	12.5	2.8		0.9					4.78	Kotrola and Conner (1997)
Turkey (mix, 11% fat)	204P		95.2	17.9	6.1		2.7					4.58	Kotrola and Conner (1997)
Turkey (mix, 3% fat)	204P		103.3	23	10.8		2.4					5.08	Kotrola and Conner (1997)
Turkey[1]	204P		74.6	10.7	4.2		1.6					4.55	Kotrola and Conner (1997)
Turkey[2]	204P		75.1	9.6	4		1.4					4.6	Kotrola and Conner (1997)
Turkey[1]	204P		77.9	20.4	8.9		1					4.95	Kotrola and Conner (1997)
Turkey[2]	204P		83.6	26.1	8.6		0.9					4.3	Kotrola and Conner (1997)
Turkey breast (3% fat)	204P		42.3	12.5	2.8		0.9						Kotrola et al. (1997)
Turkey breast (11% fat)	204P		38.5	11	2.4		6.9						Kotrola et al. (1997)
Turkey frank (17% fat)	204P		90.4	20.5	8.4		1						Kotrola et al. (1997)
Turkey ham (11% fat)	204P		60.4	16.7	4.5		1.1						Kotrola et al. (1997)
Turkey sausage (31% fat)	204P		68.8	17.8	6.7		1						Kotrola et al. (1997)

[1] With (w/t) Na lactate and 11% fat.
[2] With (w/t) Na lactate and 3% fat.

temperature of 149°C, air velocity of 7–13 m^3/min, and a wet bulb temperature of 39–98°C. They developed models to correlate cooking time and thermal inactivation of the studied pathogens with cooking conditions and found that thermal lethality of the pathogens increased with increasing the product temperature and wet bulb temperature. Osaili, Griffis, Martin, Gbur, and Marcy (2006) developed models to predict cooking time required to reach internal temperatures of 71.1°C, 73.9°C, 76.7°C, and 82.2°C in chicken leg quarters during cooking in air impingement oven. The models depend on sample weight, thickness, and initial temperature. As the thickness increased and the initial temperature decreased, more time was needed for the product to reach the desired final internal temperature. Larger samples that obviously weighed more and were thicker took more time to cook.

Steam or Hot-Water Pasteurization

Post-processing contamination of RTE meat and poultry products is a concern for the public as well as the government (Cagri, Ustunol, & Ryser, 2002). In 1998, 101 cases of listeriosis including 21 deaths were linked to the consumption of post-cooked contaminated hotdogs in the United States (CDC, 1998). To decrease the risk of post-cooking contamination, USDA-FSIS (2003) issued a final rule to control *L. monocytogenes* in establishments that produce RTE meat and poultry products exposed to the environment after lethality treatments. The aim of that rule is to prevent post-cooking contamination with the ubiquitous microorganism, *L. monocytogenes*. This rule commands the establishments to address one of three alternative strategies to control *L. monocytogenes*. Alternative 1: use both a post-lethality treatment and a growth inhibitor for *Listeria* on RTE products, Alternative 2: use either a post-lethality treatment or a growth inhibitor for *Listeria* on RTE products, Alternative 3: use sanitation actions only.

Since the potential contamination of RTE meat and poultry products with foodborne pathogens, particularly *L. monocytogenes,* presents a food safety threat, the use of post-cook heat treatment as a pre-packaging or as a post-packaging treatment has been applied to reduce the risk of contamination of RTE poultry or meat products. In a pre-packaging process, heat is applied to cooked products immediately before they are packaged while in the post-packaged process, heat is applied to cooked products after they are packaged. Both steam and hot water can be used to pasteurize the RTE meat and poultry products, however, steam seems to be used more in pre-packaging treatments.

Murphy, Duncan, Driscoll, Marcy, and Beard (2003) used hot water as a post-cook in package pasteurization to eliminate *L. monocytogenes* from unsliced RTE turkey breast meat products. In their study, the products (4 kg each) were inoculated with *L. monocytogenes* at a level of 10^7 cfu/cm^2 of the product, vacuum packaged in different thickness (0.08 mm) of packaging film then treated with water at 96°C. They found that the inactivation of the pathogen was affected by product surface roughness. About 50 min was needed to achieve

a thermal reduction of 7 logs cfu/cm^2 on product with surface roughness up to 15 mm in depth. McCormick, Han, Acton, Sheldon, and Dawson (2003) found that surface pasteurization treatment at water bath temperature of 70°C, 80°C, and 85°C for 1 min reduced the level of *L. monocytogenes* and *Salmonella* Typhimurium (10^8 cell) to below detection levels. Furthermore, it took less than 10 s at 85°C and 70°C to reduce *L. monocytogenes* and *Salmonella* Typhimurium (10^8 cell), respectively, to below detection levels in packaged low-fat ready-to-eat turkey bologna. Luchansky, Cocoma, and Call (2006) reported that hot-water pasteurization of cook-in-bag turkey breast could reduce the level of *L. innocua* on the surface of the product by 2.0 logs within 3 min at 96.1°C and 91.7°C and within 7 min at 87.8°C. Murphy and Berrang (2002) found that hot water at 88°C for 34 and 20 min could eliminate 7 $\log_{10}$ cells of *Salmonella* Senftenberg and *L. innocua* on 454 and 227 g of cooked packaged chicken breast strips, respectively. Murphy, Duncan, Johnson, Davis, Wolfe, et al. (2001) found that 34 and 28 min could eliminate the same levels of the pathogens on 450 g of the same product with continuous and batch steam process at 88°C, respectively. Murphy, Driscoll, Arnold, Marcy, and Wolfe (2003) found that steam pasteurization at 96°C for 22 min achieved 7-*D* reduction of *L. monocytogenes* in fully cooked and vacuum packaged chicken leg quarters. Muriana, Quimby, Davidson, and Grooms (2002) studied the post-package pasteurization of RTE deli meats by submersion heating for reduction of *L. monocytogenes*. A variety of RET meat products including turkey, ham, and roast beef were surface inoculated with *L. monocytogenes*, vacuum sealed in shrink-wrap and processed by submersion heating in steam-injected water bath. Processing at 90.6°C, 93.3°C, and 96.1°C for 2–10 min caused 2–4 log reduction of *L. monocytogenes* on deli meat. Gill, Thippareddi, Phebus, Mardsen, and Kastner (2001) were able to achieve 5 log reduction of *L. monocytogenes* on beef salami and turkey kielasa using steam pasteurization at 85–95°C. Cygnarowicz-Provost, Whiting, and Craig (1994) found that steam surface pasteurization at 115–136°C for 30–40 s reduced the levels of *L. innocua* on the surface of beef frankfurters by 4 logs without affecting the color and weight. Murphy et al. (2005) used a mixture of pressurized steam and hot water in an integrated pasteurization-packaging system to reduce *L. monocytogenes* from fully cooked franks. They inoculated franks with up to 6 $\log_{10}$ (CFU/cm^2) of *L. monocytogenes*, treated the inoculated samples at 121°C for 1.5 s, and observed approximately 3 logs reduction in *L. monocytogenes* regardless of the level of the initial inoculum. Murphy, Hanson, Johnson, Chappa, and Berrang (2006) reported that surface pasteurization of fully cooked frankfurters, during vacuum packaging, using steam at 114°C for 1.5 s combined with organic acid solution of 2% acetic, 1% lactic, 0.1% propionic, and 0.1% benzoic acids could reduce the level of *L. monocytogenes* by 3 logs. They reported that organic acids could inhibit the growth of the surviving during storage. Mangalassary, Dawson, Rieck, and Han (2004) found that meat bologna thickness and fat content significantly affect the heating rate and final surface temperature during in-package pasteurization of the meat.

The effect of process conditions on process lethality of meat and poultry products has been studied. Kim, Murano, and Olson (1994) found that heating rate, heating atmosphere, and meat age had an effect on survival of *L. monocytogenes* was examined in ground pork. They found that more survivors were noticed in the samples when the meat was heated at rate of 1.3°C/min compared with at 8.0°C/min and when the meat was heated aerobically than anaerobically. Furthermore, they found that the microbe was more resistant to heat in fresh ground pork compared with 3-month-old pork (*D*-value at 62°C was 7.7 min compared with 5.2 min).

Real-Time Control

The science through which investigators can characterize, for virtually any meat product, the growth rate of various microbial species and the effectiveness of thermal processing in inhibiting their growth is well developed. Techniques for applying this knowledge to processing of products on an industrial scale are still being developed.

In 1999, a team of engineers led by Dr. Yang Tao at the University of Arkansas reported on the development of a non-invasive method for estimating the internal temperature of chicken breasts right after they were cooked (Ibarra, Tao, Walker, & Griffis, 1999). They captured infrared images of the meat products as the chicken breasts emerged from an industrial oven. A series of such images over a short period of time allowed the investigators to apply an autoregressive mathematical model to estimate internal temperatures at the center of the thickest point of each product from the detected external temperatures. The investigators found that the model was able to estimate internal temperatures with an error of ±0.55 °C.

The study was conducted using chicken breasts with a similar thickness and shape. The investigators noted that this was a limitation, and that further studies would be necessary before the technique could be applied to real industrial conditions.

In 2005, Dr. Tao led another project at the University of Maryland that promised to overcome the limitations in the earlier work (Ma & Tao, 2005). The new process developed by the investigators involved an infrared imaging system, a laser range imaging system, and a neural network modeling system. Again, the infrared system was used to measure the pattern of external temperatures on chicken breasts as they emerged from an industrial oven. A series of such images was captured, in order to be able to project back in time to estimate the internal temperature of the chicken breasts when they were at their peak temperature. The laser ranging system was used with a visible light video camera to estimate the three-dimensional shape of each chicken breast.

There is great promise that advanced technology, such as that described above, can lead to systems in which each individual meat product is inspected and only those that are guaranteed to be safe will be shipped to market.

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Chapter 10
Antimicrobials Treatment

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Introduction

The use of antimicrobials is a common practice for preservation of foods. Incorporation, in a food recipe, of chemical antimicrobials towards inhibition of spoilage and pathogenic micro-organisms results in the compositional modification of food. This treatment is nowadays undesirable for the consumer, who likes natural products. Scientific community reflecting consumers demand for natural antimicrobials has made efforts to investigate the possibility to use natural antimicrobials such us bacteriocins and essential oils of plant origin to inhibit microbial growth.

In addition, to the compositional modification of a food, antimicrobials are also used for a food surface treatment or for incorporation in the packaging material. This is especially important for cooked meat products, to decontaminate them from post-thermal processing cross-contamination. Antimicrobial substances are also used in certain stages of food process corresponding to critical control points; their presence contributes to the safety design of a food with other existing hurdles of microbial growth.

In this chapter natural (bacteriocins and essential oils) and chemical antimicrobials used in meat and meat products processing are reviewed providing in parallel basic information on antimicrobials and factors affecting their use in foods.

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Bacteriocins

Lactic acid bacteria are widely used in fermented foods such as dairy, meat, vegetables and bakery products. The frequent use of lactic acid bacteria in foods, usually as starter cultures, is owned to the desired changes that induce flavour, odour and texture of the products as well as that positively contribute to the products' safety because they inhibit the growth of pathogens. Antimicrobial activity of lactic acid bacteria is due to pH decrease, microbial competition for nutrients and production of antimicrobial compounds such as hydrogen peroxide, lactic acid and other metabolites (e.g. bacteriocins) (Ray & Daeschel, 1992).

Bacteriocins are proteinaceous compounds, consisting of peptides and amino acids, with antimicrobial activity and are synthesized by the ribosomes of the microbial cells. Over the last decades, numerous bacteriocins produced by lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus* and *Carnobacterium*) have been isolated and characterized (nisin, lactococcins, sakacins, curvaticins, carnobacteriocins, pediocins, etc.) (Nettles & Barefoot, 1993; Xiraphi et al., 2006). Bacteriocins gained increased attention because of their potential application as natural antimicrobials in foods to substitute or decrease the addition of other chemical preservatives which are considered dangerous (e.g. nitrites). On the other hand, bacteriocins are generally considered as GRAS (generally regarded as safe) substances and thus, may be added to foods or produced in situ aiming to act as natural preservatives. Nowadays, only nisin has been approved as additive and produced in industrial scale in a semi-purified form for use in dairy products (EC, 1983; Parente & Ricciardi, 1999; FDA, 2008).

Classification of Bacteriocins

Bacteriocins are extracellular compounds displaying a relative narrow antimicrobial spectrum. Tagg, Dajani, and Wannamaker (1976) termed as bacteriocins the substances that are produced by the Gram-positive bacteria and have the following properties: their molecule consist of peptides which have antimicrobial activity, they have low molecular weight which vary between the different bacteriocins as their antimicrobial spectrum and mode of action, and in general are active against close-related species to the producing strain. Bacteriocins are classified into four categories (Klaenhammer, 1993; Nes et al., 1996):

– Class I. Lantibiotics belong to this group. These are of low molecular weight peptides (<3.5 kDa) resistant to thermal treatment and are characterized by the presence of uncommon amino acids such as lanthionine and 3-methyl-lanthionine. The major representative bacteriocin from this class is nisin. Lantibiotics are divided into the following sub-categories: spiral positively

charged molecules of low molecular weight (2151–4635 Da) and circular neutral or negatively charged molecules with even lower molecular weight (1825–2042 Da).
- Class II. Low molecular weight bacteriocins (<10 kDa) with 30–100 amino acids resistant to heat without lanthionine. This class is organized into three sub-groups: The IIa sub-group is the most common group including peptides active against *Listeria* strains. Although the bacteriocins of this group are not so active against spores are more efficient than nisin in some food categories such as meat. The most significant representative of this sub-group is pediocin PA-1/AcH. The IIb sub-group includes bacteriocins that consist of two peptides (lactococcin G) and the IIc sub-group requiring reduced cystein molecules for activity expression (lactococcin B).
- Class III. Macromolecular bacteriocins (molecular weight >30 kDa) not so stable to heat and are inactivated at high temperatures (helveticin J).
- Class IV. Bacteriocins of this category contain in their molecule a carbohydrate or fatty part needed for their activity expression (lactocin 27).

More recently, Cotter, Hill, and Ross (2005) proposed a revised classification scheme including the Classes I (lanthionine-containing lantibiotics) and II (non-lanthionine-containing bacteriocins as well as circular bacteriocins) and bacteriolysins (formerly class III). Class IV was not included in the new designation scheme because, according to the authors, bacteriocins that require non-proteinaceous moieties for activity have not yet been convincingly demonstrated.

Mode of Action, Molecular and Biochemical Characterization of Bacteriocins

Bacteriocin production is regulated by genes responsible for the production (Bac^+) of the substance and immunity of the producer strain (Bac^r) to its own antimicrobial compound. Recently, research has been driven to the isolation and transfer of genes related to bacteriocin production of wide antimicrobial spectrum from one strain to another, incapable of producing bacteriocin or producing bacteriocin with narrow antimicrobial activity (Ennahar, Sashihara, Sonomoto, & Ishizaki, 2000). Moreover, synthesis of these antimicrobial agents is regulated by four different genes (Nes et al., 1996):

- the gene responsible for the production of a precursor substance of the bacteriocin
- the gene which provides immunity to the producer strain
- the gene encoding the transport of the precursor bacteriocin outside the cell activating an ad hoc mechanism for this purpose (ABC-transporter) and finally

– the gene which activates the mechanism of production of a supplementary auxiliary protein (accessing protein) important for the extracellular transport and activation of the bacteriocin, yet its exact role is still unknown.

Besides these genes, more genes have been found for some bacteriocins of Class II responsible for the regulation of the bacteriocin production (Diep, Havarstein, & Nes, 1996).

Bacteriocins are firstly synthesized inside the ribosomes as precursor substances (pro-peptides) which are biologically inactive containing an amino acids sequence (number of amino acids vary between 14 and 30) characterized by the presence of two molecules of glycine (double-glycine leader). This sequence serves a dual purpose: to prevent the expression of the bacteriocin activity while it is still present inside the producer cell and to signal for the activation of the ABC-transporter and accessing protein mechanisms (Klaenhammer, 1993). The mechanism of bacteriocin synthesis regulation may be explained by the quorum sensing phenomenon (Cotter et al., 2005) and consists of three elements: the induction factor (IF) and two proteins, the histidine protein kinase (HPK) and the response regulator (RR). The IF, a bacteriocin-like peptide without antimicrobial activity, is required for the stimulation/activation of the genes encoding for bacteriocin synthesis. This is observed for bacteriocins belonging to Class II, whereas for lantibiotics and non-lantibiotics the bacteriocin itself serves as external signal to regulate bacteriocin synthesis (Chen & Hoover, 2003). The N-terminal of the kinase serves as receiver of the IF and the signal is transmitted to its C-terminal leading to the activation of the enzyme and the initiation of the histidine phosphorylization. For lantibiotics and non-lantibiotics, the HPK phosphorylates the histidine residue when it senses a certain level of bacteriocin concentration in the environment (Chen & Hoover, 2003). The latter (kinase is also involved) facilitates the phosphorylization of the aspartic acid of the RR protein. The last phosphorylization causes changes in the carbon part of the RR protein activating the transcription of the corresponding genes (pre-peptide synthesis, transportation, immunity and in some cases bacteriocin production regulation) (Nes et al., 1996). The proteins involved in bacteriocin transportation (ABC-transporter system) have two regions, one hydrophobe and one carbon region for ATP binding (ATP-binding region) which have essential role during transportation. It has been proved that these proteins also carry one N-terminal extension consists of 150 amino acids with potential proteolytic activity to selectively breakdown the double-glycine leader sequence of the pre-peptides. As previously mentioned, accessory protein (470 amino acids) is also required which is believed to facilitate bacteriocin transportation and/or cleavage of the double-glycine leader sequence. However, the precise role of this protein has not yet been fully elucidated (Havarstein, Diep, & Nes, 1995).

Immunity of the bacteriocin-producing strains to their own bacteriocin is regulated by the corresponding gene encoding the extraction of immunity-related proteins. Such proteins are the LciA (encoded by the *lciA* gene) and

NisI (encoded by the *nisI* gene) offering protection against lactococcin A and nisin, respectively. These proteins deactivate the produced bacteriocin reacting (binding of the proteinic receivers of the bacteriocins) with the bacteriocin molecules (Ennahar et al., 2000).

Mode of action of the bacteriocins is characterized by two distinct phases: (1) adsorption of the bacteriocin on receivers located on the cell wall of the sensitive strains and (2) denaturation of the cytoplasmic membrane. The first step is reversible and removal of the bacteriocin (e.g. presence of proteases) maintains membrane structure intact without any damage of the bacterial cells. The second step is irreversible and the damages caused are characteristic for each bacteriocin (pore formation, lytic action or synthesis inhibition of important cell components). Cytoplasmic membrane is the main target of the bacteriocin causing extensive leakage of ions and other important cell components such as ATP or amino acid and also blockage of amino acids transportation inside the bacterial cell (Abee, Krockel, & Hill, 1995). In general, bacteriocin activity is the result of the hydrophobic and electrostatic interactions between the positively charged bacteriocins and negatively charged cytoplasmic membrane (due to the presence of many negatively charged lipids) of the sensitive strains (Ganzle, Weber, & Hammes, 1999; Moll, Konings, & Driessen, 1999).

Bacteriocins are mainly active against Gram-positive close-related species (e.g. *Listeria monocytogene*s). Gram-negative strains (e.g. *Salmonella* spp.) are more resistant to bacteriocins of the lactic acid bacteria because their membrane composition differs from that of the Gram-positive micro-organisms. Cytoplasmic membrane of the Gram-negative micro-organisms is characterized by the presence of an additional external layer, containing phospholipids, proteins and polysaccharides lipids (LPS), impermeable to most substances. The layer facilitates the diffusion of molecules with molecular weight below 600 Da, whereas the lowest in size bacteriocin found has molecular weight approximately 3 kDa. However, the presence of various agents such as EDTA or citric acid makes the membrane sensitive to bacteriocins because of Mg^{2+}-binding in the LPS layer of the external membrane (Stevens, Sheldon, Klapes, & Klaenhammer, 1991).

Applications of Bacteriocins in Foods

Lactic acid bacteria are widely used for food preservation because they inhibit the growth of various pathogenic bacteria lowering the pH during fermentation and producing antimicrobial substances. Bacteriocins constitute a group of such antimicrobials which may find application in dairy, meat, fishery, bakery and vegetable products as well as in alcoholic beverages production for controlling pathogenic and in some cases spoilage micro-organisms (non-starter lactic acid bacteria in cheese and wine) (Daeschel, Jung, & Watson, 1991; O'Sullivan, Ross, & Hill, 2003). Bacteriocins may be applied as food preservatives directly or indirectly. Direct method is referred to the addition of bacteriocins to foods in purified/semi-purified form or incorporated into packaging film surfaces

(Appendini & Hotchkiss, 2002), whereas the addition of micro-organisms producing bacteriocins in situ is known as indirect method (Luchansky, 1999). Bacteriocins have gained an interest as food preservatives due to the preference of consumers for safe foods with an extended shelf life but minimally processed without excess of chemical preservatives. Nisin (nisaplin, Danisco) and pediocin PA1/AcH (ALTA 2431, Quest) have been widely used in foods. Hurdle technology could be employed to extend the relatively narrow antimicrobial spectrum of the bacteriocins (Chen & Hoover, 2003). Bacteriocins may be combined with other preservative techniques (e.g. high pressure or temperature shock) or chemicals (e.g. EDTA) to include Gram-negative micro-organisms (wider antimicrobial spectrum of activity) (Stevens et al., 1991; Kalchayanand, Sikes, Dunne, & Ray, 1998; Masschalck, Deckers, & Michiels, 2003).

Dairy Products

Nitrates are commonly used to inhibit the growth of clostridia causing problems during cheese making such as *Clostridium tyrobutyricum*. *L. monocytogenes* is another micro-organism of concern, especially for cheese such as Taleggio or Mozzarella, due to pH increase during ripening. Bacteriocins and more specifically nisin have been extensively used as alternatives for nitrate salts (Hugenholtz & de Veer, 1991; Giraffa, Picchioni, Neviani, & Carminati, 1995; Stecchini, Aquili, & Sarais, 1995; Ross et al., 1999).

Meat Products

Due to the successive application of the nisin in dairy products, an interest was developed for substitution of nitrate/nitrite salts by nisin during meat products manufacturing (Rayman, Aris, & Hurst, 1981; Taylor, Somers, & Krueger, 1985). However, the results were not as encouraging as dairy products due to low solubility of the nisin in the increased pH of the meat products (Rayman, Malik, & Hurst, 1983; Stiles, 1996; Schillinger, Geisen, & Holzaphel, 1996). Better results were obtained by the application of bacteriocin-producing micro-organisms isolated from meat products such as *Pediococcus*, *Leuconostoc*, *Carnobacterium* and *Lactobacillus* spp. Also, various bacteriocins such as sakacin, pediocin, curvaticin or mesenterocin have been added to meat products (bologna, frankfurters and ham-type meat products) in purified/semi-purified form with promising results. These bacteriocins displayed anti-listerial activity and their addition inhibited or even reduced the growth of *L. monocytogenes* (Berry, Hutkins, & Mandigo, 1991; Hugas, Pages, Garriga, & Monfort, 1998; Ross et al., 1999; Laukova, Czikkova, Laczkova, & Turek, 1999; Mataragas, Drosinos, & Metaxopoulos, 2003; Drosinos, Mataragas, Kampani, Kritikos, & Metaxopoulos, 2006).

Fishery Products

An interesting bacteriocin application is the preservation of shrimps in brine (3–6% NaCl). Usually these products are preserved by the addition of sorbic or benzoic acids. Bacteriocins produced by *Lactococcus lactis* SIK-83 (nisin Z), *Carnobacterium piscicola* U149 (carnocin U149) and *Lactobacillus bavaricus* MI401(bavaricin A) prolonged the shelf life by 21 days (nisin), 6 days (bavaricin) or the shelf life was similar (carnocin) with the control (10 days) without the addition of benzoate or sorbate solutions. However, best results were obtained with the use of antimicrobials (59 days) (Einarsson & Lauzon, 1995). Moreover, other bacteriocins (e.g. nisin and sakacin) alone or combined with other hurdles (e.g. low temperature, modified atmosphere and antimicrobials such as lactate or carbon dioxide) have been studied to investigate the growth of pathogenic bacteria (e.g. *L. monocytogenes*) in cold-smoked salmon and rainbow trout (Nilsson, Huss, & Gram, 1997; Nykanen, Weckman, & Lapvetelainen, 2000; Katla et al., 2001).

Fermented Vegetables

Bacteriocin-producing lactic acid bacteria may be applied in products of plant origin such as fermented vegetables (sauerkraut). Salt, acetate and sugar are frequently used in this kind of products to inhibit the growth of undesired micro-organisms. Anti-listerial bacteriocins (sakacin A and pediocin), produced by *L. sakei* Lb706 and *Pediococcus acidilactici* M, respectively, are used during Kimchi manufacturing and the results showed that the former bacteriocin did not inhibit *L. monocytogenes* whereas pediocin readily reduced the population during fermentation at 14°C (Choi & Beuchat, 1994).

Factors Limiting Bacteriocins Efficiency in Foods

Studies have shown that bacteriocins are not so effective in foods compared to laboratory substrates. This is attributed to the fact that these studies, performed in laboratory media, have been carried out under controlled conditions without any interference as frequently happens in foods. Foods are complex systems consisting of various microenvironments which interact with each other. Interactions between bacteriocin molecules and food ingredients may negatively contribute to bacteriocin efficiency. Bacteriocins (e.g. nisin or pediocin) may initially reduce bacterial counts; however, initiation of growth occurs during storage, after an extended lag phase. Factors that are likely to interact with bacteriocins resulting in decreased activity are summarized below (Schillinger et al., 1996):

- Acidity (pH) may influence activity, solubility and stability of the bacteriocins
- Low solubility of the bacteriocins resulting in inadequate and non-homogenate diffusion of the substances inside the food mass
- Bacteriocin binding from various food ingredients such as fat molecules
- Inactivation of the bacteriocin molecules by other food additives
- Presence of various enzymes that breakdown bacteriocins such as proteases
- Mechanisms such as lipid oxidation destabilizing bacteriocin molecules.

Future Considerations on Bacteriocins Applications in Foods

When bacteriocin application is combined with conventional preservation techniques and good hygiene practices (GHP), pathogenic bacteria or spoilage microorganisms may be effectively controlled. However, the addition of bacteriocins in purified form is not used by food industries in extensive scale because of the high cost of this application. Bacteriocin addition as additives comprises an attractive alternative solution for minimally processed foods to ensure their safety. Also, bacteriocins may be used as substitutes of chemical preservatives such as nitrite/nitrate salts and sorbate/benzoate. Bacteriocin efficiency or spectrum activity may be increased by combining bacteriocins with other substances (e.g. chelators) or new preservation techniques (e.g. Ultra Hydrostatic Pressure and Pulsed Electric Field) which may lead to substitution of some chemicals or to the application of milder methods of processing (e.g. thermal treatment). Various molecular techniques (e.g. transfer of genes responsible for bacteriocin production to other non-bacteriocin-producing strains, mutation of genes responsible for bacteriocin production, technology of protein engineering, etc.) may be employed to develop proteinic molecules with improved solubility and stability, broader antimicrobial spectrum and higher antimicrobial activity. Furthermore, these techniques may serve as means to develop bacterial strains capable of producing such improved proteinic molecules (Abee et al., 1995).

Recently, mathematical models have been developed to describe growth and bacteriocin production of bacteriocin-producing strains added (Messens, Neysens, Vansieleghem, Vanderhoeven, & De Vuyst, 2002; Messens, Verluyten, Leroy, & De Vuyst, 2003). Modelling contributes to the determination of how environmental factors affect the growth and bacteriocin production and also to predict the bacteriocin efficiency (Leroy, Verluyten, Messens, & De Vuyst, 2002; Leroy & De Vuyst, 2003). Commercial use of bacteriocins requires optimization of their production in order to make cost-effectively their application. One method that is usually followed for the optimization of bacteriocin production is by varying one factor in turn while the other factors are kept constant. This method is laborious and requires a lot of time in case of that several factors are under study. Hence, statistical experimental designs have been developed to evaluate the influence of substrate composition and

environmental conditions (e.g. temperature and/or pH) on growth and bacteriocin production (Rollini & Manzoni, 2005; Dominguez, Bimani, Caldera-Olivera, & Brandelli, 2007).

Naturally Occurring Compounds from Plants

Nowadays, there is an increasing demand worldwide for environmental friendly and more natural antimicrobials to be used for mild preservation. This is due to the negative attitude of consumers towards preservatives of chemical origin. Nature, and especially plants, has been recognized as a remarkable source of antimicrobial compounds, which are primarily intended to increase natural preinfectional and postinfectional defence of plants against micro-organisms and insects (Smid & Gorris, 1999). Such systems include prohibitins, inhibitins, postinhibitins, phytoalexins, phenolics and essential oils. Over 1300 plants are considered as potential sources of antimicrobials (Nychas, 1995). Phenolics and essential oils (EOs) are the major compounds which have also been proven promising for food preservation, since the 1920s (Shelef, 1983). They are commonly obtained by steam- or hydro-distillation as well as by solvent extraction (e.g. with ethanol) from spices and herbs (Davidson & Naidu, 2000; Coma, 2008).

Since ancient times (with the earliest report in 1550 BC), spices and herbs have been used for their perfume and flavour as seasoning additives and as preservatives due to their strong antimicrobial and antioxidant properties (Tassou, Lambropoulou, & Nychas, 2004; Coma, 2008). Herbs are distinguished from spices in that herbs commonly constitute portions of aromatic, soft stemmed plants and aromatic shrubs and trees, whereas spices are rhizomes, roots, barks, flower buds, fruits and seeds (Davidson & Naidu, 2000). The antimicrobial activity of spices and herbs is primarily attributed to the phenolic component of their essential oil fraction (phyto-phenols; Davidson & Naidu, 2000). In particular, essential oils mainly consist of terpenes (e.g. monoterpenes, sesquerpitenes), terpenoides and other aromatic compounds (e.g. simple phenols, such as eugenol and thymol, aldehydes, esters and alcohols) (Davidson, 1997; Smid & Gorris, 1999; Bakkali, Averbeck, Averbeck, & Idaomar, 2008). Other plant extracts include isothiocyanate derivatives (e.g. found in cabbage, horseradish, mustard, broccoli) and phenolic compounds, such as di- or tri-phenols, phenolic acids, such as hydroxucinnamic acid, and flavonoids (Davidson, 1997). Based on toxicological studies, the majority of active components of herbs and spices are considered as food-grade or generally recognized as safe (GRAS) (Smid & Gorris, 1999). Various mechanisms of inhibition have been suggested for essential oils, damaging structural and functional properties of bacterial membranes being the most dominant. In particular, EOs penetrate cell envelope, dissolve in the lipid layer of cellular membranes, bind to the hydrophobic sites of membrane proteins, and by increasing the

permeability of the cell membrane, they cause loss of vital intracellular material or inhibit nutrients intake via dissipation of pH gradient and the electrical potential (compounds of proton motive force; Tassou et al., 2004; Burt, 2004; Nychas & Skandamis, 2005; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2006). Additional modes of action include inhibition of oxygen uptake, inhibition of nucleic acid synthesis and inactivation of membrane proteins (e.g. ATPase) or other intracellular enzymes (Lemay et al., 2002; Burt, 2004). Gram-positive bacteria are considered more susceptible to EOs than Gram-negative bacteria (Smith-Palmer, Stewart, & Fyfe, 1998; Fisher & Phillips, 2006). Of the Gram-positive bacteria, lactic acid bacteria have been reported as the most resistance to EOs (Ouattara, Simard, Piette, Bégin, & Holley, 2000; Lemay et al., 2002).

Factors Affecting the Effectiveness of Essential Oils in Foods

Even though the results of most in vitro assays suggest that essential oils have a substantial antimicrobial effectiveness, when used in food systems, the amounts required are considerably higher (10- to 100-fold) or the concentration of the targeted micro-organisms quite lower (Shelef, 1983; Burt, 2004). Given that effective levels in foods may often have negative sensory impact, the commercial application of EOs in foods is currently limited. For instance, when oregano or nutmeg EOs were added at the maximum acceptable organoleptic level on cooked chicken breast they showed limited activity even at refrigeration temperatures (Firouzi, Shekarforoush, Nazer, Borumand, & Jooyandeh, 2007). The performance of EO in foods is the additive (potentially synergistic) or antagonistic outcome of several factors, and specifically: (i) certain intrinsic properties of foods, such as fat, pH, salt, water and proteins, which determine the solubility of EOs in the water phase (Kabara, 1991; Juven, Kanner, Schved, & Weisslowicz, 1994; Nychas, 1995; Smith-Palmer, Stewart, & Fyfe, 2001); (ii) the structure and viscosity of the foods (solid vs. liquid foods) (Skandamis, Tsigarida, & Nychas, 2000); (iii) the decomposition of some EOs constituents (e.g. allyl isothiocyanate) in aqueous face and/or their interaction with certain hydrophilic substances, such as thiols, and sulphydryl or terminal amino groups of proteins (Ward, Delaquis, Holley, & Mazza,, 1998); and (iv) factors affecting the physiology of the micro-organisms, such as composition of bacterial membranes, availability of nutrients, oxygen tension and incubation temperature (Kabara, 1991; Juven et al., 1994; Smid & Gorris, 1999; Gill, Delaquis, Russo, & Holley, 2002; Nychas & Skandamis, 2005).

The major limiting factor for the activity of EOs in foods, even when they are applied at concentrations highly above those required for inhibition based on in vitro studies, is their reduced solubility, due to the presence of apolar constituents in their composition. For instance, highly hydrophobic constituents of EOs (e.g. thyme, mint and bay oil) may show limited effectiveness in foods of high fat

content, such as liver pate (30–40%), full-fat cheese or adipose meat tissue, because EOs will partition in the lipid fraction of food phase, thereby reducing the residual EO concentration in the hydrophilic portion where micro-organisms are partitioned (Tassou, Drosinos, & Nychas, 1995; Cutter, 2000; Smith-Palmer et al., 2001; Holley & Patel, 2005). However, the opposite may also occur with less hydrophobic EOs, such as clove oil, which was more effective against *L. monocytogenes* and *S. enteritidis* in full-fat than in low-fat cheese (Smith-Palmer et al., 2001). In this respect, the octanol/water partitioning coefficient of an EO (Smid & Gorris, 1999) may be a reliable indicator of the expected antimicrobial effectiveness. Furthermore, the activity of EOs may be quenched by other macromolecules which form hydrophobic cavities and hydrogen bonds, as exemplified by Tween 80 (Juven et al., 1994). Low pH increases the hydrophobicity of essential oils, enhancing their potential to bind onto hydrophobic sites of membrane proteins, and EOs also become more soluble in the lipid-rich membranes of the target micro-organisms (Juven et al., 1994). Nevertheless, low pH may also increase the solubility of the EOs in the lipid phase and hence counteract the antimicrobial effectiveness. Furthermore, given that the phenolic group may be active both as un-ionized (e.g. at pH <5.0) and ionized, it has been suggested that phenolic preservatives may maintain effectiveness over a wide range of pH values, such as pH 3.5–8.0 (Kabara, 1991).

Apart from pH and fat, the high amounts of protein and the reduced water content of foods may also decrease the effectiveness of EOs (Burt, 2004). Complex formation between EOs constituents and proteins (e.g. bovine serum albumin or casein up to 6%) may reduce the probability of EO to attack the target micro-organisms, as exemplified in cheese and broth (Rico-Munoz & Davidson, 1983; Juven et al., 1994; Smith-Palmer et al., 2001). Furthermore, low water content may hamper the transfer of EO to the active sites in the microbial cells (Smith-Palmer et al., 2001). In this respect, a comparative evaluation of 0.03% oregano essential oil against *Salmonella typhimurium* in broth and within solid medium containing 20% gelatin showed that the counts and metabolism of the bacterium were considerably suppressed in liquid culture compared to gelatin medium (Skandamis et al., 2000). Thus, it may be postulated that broth facilitated contact of EO with *Salmonella*. Recent evidence suggested that the strong attachment of pathogens on the rough surface of chicken skins accounted for the limited effectiveness of citral, linalool and bergamot oil added at the minimum inhibitory concentrations according to in vitro data (Fisher & Phillips, 2006). It has also been speculated that the greater nutrient availability of foods compared to laboratory media may increase bacterial resistance to antimicrobial agents, including EOs (Gill et al., 2002).

Lowering oxygen tension in packages increases the effectiveness of EOs. Juven et al. (1994) showed that anaerobic conditions significantly enhanced the antimicrobial activity of 350 μg/ml thyme essential oil, 140 μg/ml thymol or 200 μg/ml carvacrol against *S.* Typhimurium on nutrient agar. This was associated either with the oxidation of phenolic constituents of EOs or the lower

energy yields of bacterial metabolism under aerobic conditions (Juven et al., 1994). The fact that 0.8% oregano essential oil in ground meat caused more pronounced inhibition to *L. monocytogenes*, *S.* Typhimurium and spoilage flora under 40% CO_2/30% O_2/30% N_2 and 100% CO_2 compared to that observed in aerobic packages supports the above explanations (Skandamis & Nychas, 2001a; Skandamis, Tsigarida, & Nychas, 2002; Tsigarida, Skandamis, & Nychas, 2000). In addition to oregano essential oil, the activity of coriander oil against *Aeromonas hydrophila* in cooked pork was significantly enhanced under vacuum as compared to aerobic storage at 10°C (Stecchini, Sarais, & Milani, 1993).

Regarding the effect of temperature, it is suggested that low temperatures reduce the activity of phenolic preservatives, either lowering the solubility of phenolics in the lipids of cell membrane or due to reduction in the rate of interaction with the membranes (Kabara, 1991). Reports by Tassou et al. (1995) on the application of mint essential oil in tzatziki (pH 4.5) at 4 and 10°C, against *L. monocytogenes*, or by Skandamis and Nychas (2001b) on the application of oregano essential oil in eggplant salad (pH 4.0–5.0) at 0–15°C, against *E. coli* O157:H7, and by Stecchini et al. (1993) for clove and coriander against *A. hydrophila* on cooked pork support these hypotheses. However, at growth-permitting conditions, it is expected that low temperature would enhance activity of EOs by delaying bacterial growth, as compared with higher temperatures (Hao, Brackett, & Doyle, 1998a, 1998b; Nadarajah, Han, & Holley, 2005a; Solomakos, Govaris, Koidis, & Botsoglou, 2008). Finally, salt (e.g. 3%) may have a potentiating effect on some phenolic compounds, as has been shown for butylated hydroxyanisole, a well-known antioxidant agent (Kabara, 1991).

Applications of EOs in Meat and Meat Products

Direct Application of EOs in the Product

The majority of studies evaluating the antimicrobial activity of phenolic compounds, essential oils or their constituents are performed in vitro. Evidence on their activity in perishable foods, such as meat and meat products, is essential in order to establish their use. An overview of pertinent studies is provided in Table 10.1, whereas major issues and outcomes of these studies are detailed in the next paragraphs.

A total of nine essential oils (20% in alcohol), namely angelica root, banana puree, bay leaf, caraway seed, carrot root, eugenol (from clove), marjoram, pimento leaf and thyme were evaluated for their ability to inhibit growth of *A. hydrophila* and *L. monocytogenes* on cooked beef (internal temperature of 74°C) and chicken (internal temperature 85°C) at 5 and 15°C (Hao et al., 1998a,

Table 10.1 Effectiveness of essential oils or their components applied directly in foods

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Eugenol Pimento leaf Caraway seeds	Cooked chicken	0.1 ml (of 20% solution in ethanol) on 25 g of slice	5, 15°C	*L. monocytogenes* 10 and 10^5 CFU/g	*L*	–	Hao et al. (1998a)
				A. hydrophila	*M–H*	–	
Eugenol Pimento leaf	Cooked beef	0.1 ml (of 20% solution in ethanol) on 25 g of slice	5, 15°C	*L. monocytogenes* 10 and 10^5 CFU/g	*N–L*	–	Hao et al. (1998b)
				A. hydrophila 10 and 10^5 CFU/g	*L–H*	–	
Mustard	Acidified chicken meat model (sausage; pH 5.0, aw>0.96, 190 ppm nitrite, cooked at 55°C)	0.1%	22°C Inoculum: 10^3–10^4 CFU/g	*Escherichia coli*	–	1.5 logs	Lemay et al. (2002)
				Brochothrix thermosphacta	*L*	–	
				Lb. alimentarius, lactic acid bacteria	*L*	–	

Table 10.1 (continued)

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Rosemary	Pork liver sausage	0.5% ground rosemary	5°C	*L. monocytogenes* Inoculum: 10^2–10^3 CFU/g	*L–M*	–	Pandit & Shelef (1994)
		1% EO			*L–M*	–	
		5% encapsulated EO (spray dried on modified starch)			*H*	–	
		0.1–0.5% antioxidant extract by CO_2 extraction			*H*	–	
Sage	Chicken noodles and strained beef	Up to 2.5% rubbed sage	35°C Inoculum: 10^5–10^6 CFU/g	*B. cereus*	*H*	–	Shelef et al. (1984)
				S. aureus	*H* in chicken *N* in beef	–	
				Pseudomonas sp.	*M* in chicken *N* in beef	–	
				S. typhimurium	*N*	–	
Clove	Minced mutton	0.5%, 1%	7, 30°C	*L. monocytogenes*	*N–L*	–	Vrinda-Menon & Garg (2001)
Clove	Cooked pork (75°C for 30 min)	500 μg/cm^2	2°C; air or vacuum	*A. hydrophila* Inoculum: 10^6 CFU/cm^2	–	2–3 logs within 8 days	Stecchini et al. (1993)
			10°C; air or vacuum		Growth in the control	4–6 logs within 8 days	

Table 10.1 (continued)

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Coriander	Cooked pork (75°C for 30 min)	1250 μg/cm^2	2°C; air or vacuum	*A. hydrophila* Inoculum: 10^6 CFU/cm^2	–	4 logs within 8 days	
			10°C; air or vacuum		Growth in the control	6 logs within 8 days	
Mint	Pâté	0.5–2%	4, 10°C Inoculum: 10^7 CFU/g	*L. monocytogenes*	*N–L*	–	Tassou et al. (1995)
				S. enteritidis	*N–L*	–	
Thyme	Minced pork	Up to 0.25%	4, 8°C	*L. monocytogenes* Inoculum: 10^4 CFU/cm^2	*H*	2–2.3 logs initial reduction	Aureli, Costantini & Zolea (1992)
Oregano	Minced pork	Up to 0.4 μl/g	Vacuum with or without nitrites	*C. botulinum* spores Inoculum: 300–3000 spores/g	*N* without nitrites	–	Ismaiel & Pierson (1990)
Garlic	Fresh sausage Beef burger	3–5% ground garlic	25°C	*S. typhimurium* Inoculum: 10^4 CFU/cm^2	*M–H*	–	El-Khateib & El-Rahman (1987)
Clove	Beef	1% dry mass	4, 24°C	*L. monocytogenes*	–	1 log only at 24°C	Ting & Deibel (1992)
Oregano	Minced beef	0.05–1%	5°C – Air – 40% CO_2 / 30% O_2 / 30% N_2 – 100% CO_2	Spoilage meat flora	*N* in air *M* in $CO_2/O_2/N_2$ *H* in CO_2	–	Skandamis et al. (2001a)

Table 10.1 (continued)

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Oregano	Beef fillets (i) With natural flora (ii) Flame sterilized	0.8%	5°C under – Air – Vacuum – 40%CO_2/30%O_2/30%N_2 Films of low and high permeability	Spoilage flora *L. monocytogenes* *S. typhimurium* Inoculum: 10^3 CFU/g	*L* in air *M* in vacuum/MAP-high permeability *H* in vacuum/MAP-low permeability	1 log reduction of pathogens in flame sterilized fillets	Skandamis et al. (2002) Tsigarida et al. (2000)
Protecta II (herbal mix)	Chicken broilers	2%	Spray chilling (1°C, 30 min)	Natural flora	–	2–3 logs	Dickens (2000)
Bell pepper (*Capsicum annuum* extract)	Minced beef	0.02–2.5 ml/100 g minced meat 0.02–5 ml/100 g minced meat	7°C with 0–4% NaCl	*S. typhimurium* Inoculum: 10^3 CFU/g *P. aeruginosa* Inoculum: 10^3 CFU/g	*H* at 1.5 ml/100 g *H* at 0.3 ml/100 g	3 logs at >1.5 ml/100 g 3 logs at >1.5 ml/100 g	Careaga et al. (2003)
Thyme	Minced beef (Flame sterilized)	0.6%	4, 10°C alone or with nisin	*L. monocytogenes* (2-strains cocktail) Inoculum: 10^4 CFU/g	*H*	3 logs initial reduction	Solomakos et al. (2008)
Savory Oregano	Pork fillets	50 µl/100 g meat	4°C alone or with *L. curvatus*	*L. monocytogenes* Inoculum: 10^4 CFU/g	–	3 logs	Ghalfi et al. (2007)

Table 10.1 (continued)

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Oregano	Beef patties	200–500 ppm	2°C with ascorbic acid and lycopene, 70%O_2/ 20%CO_2/ 10%N_2	Natural flora	*M*	–	Sánchez-Escalante et al. (2003)
Rosemary	Beef stakes	0.1% (1000 ppm)	1°C with lactic acid: – 70%O_2/ 20%CO_2/ 10%N_2 – 60%O_2/ 40%CO_2	Natural flora	*H*	–	Djenane et al. (2003a, b)
Oregano	Chicken	0.1%, 1%	4°C – Air – 30%CO_2/ 70%N_2 – 70%CO_2/ 30%N_2	Natural flora	*H*	–	Chouliara et al. (2007)
Oregano Nutmeg	Barbecued chicken breast	1–3 μl/g	3, 8, 20°C Inoculum: 10^6 CFU/g	*Yersinia enterocolitica* *L. monocytogenes*	*N*	–	Firouzi et al. (2007)
Cilantro oil	Ham	0.1–6% in canola oil	10°C vacuum Inoculum: 10^4 CFU/cm^2	*L. monocytogenes* (5-strains cocktail)	*N*	–	Gill et al. (2002)

Table 10.1 (continued)

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Protecta I, II (herbal mixtures)	Lean beef tissues	Spray (15 s) with 2.5% Protecta I and II	4°C Inoculum: 10^5 CFU/cm^2	*E. coli* O157:H7 *L. monocytogenes* *S.* Typhimurium	*M*	1 log initial reduction	Cutter (2000)
	Ground beef (lean and adipose)	Mixed with 2.5% Protecta I and II liquid, powder, spray		Aerobic plate counts	*N–L*	–	
Clove	Buffalo meat steaks	Dipped in 0.1%	4°C with 2% lactic acid and Vitamin C	Natural flora	*L–M*	–	Naveena et al. (2006)
Bergamot Citral Linalool	Chicken skin	Dipped in oils at MIC[e] for 15–60 s	Room temperature for 60 s Inoculum: 10^8 CFU/g	*E. coli* O157:H7 *L. monocytogenes* *S. aureus* *B. cereus*	–	1–3 logs	Fisher & Phillips (2006)
Mustard flour	Ground beef	5%, 10%, 20%	4°C, 100% N_2	Natural flora *E. coli* O157:H7 (5-strains cocktail) 10–100, 10^3, 10^6 CFU/g	*N*	– Elimination Elimination No at 5%, 10% Elimination at 20%	Nadarajah et al. (2005a)

Table 10.1 (continued)

Antimicrobial agent or plant essential oil	Food	Concentration[a]	Experimental conditions[b]	Microorganism	Growth inhibition[c]	Inactivation[d]	References
Carvacrol	Steak tartare	5 mmol/g	10°C	*L. monocytogenes* Inocula 10^7 CFU/g	*N*	–	Veldhuizen et al. (2007)
Carvacrol Thymol Oregano Cinnamal-dehyde	Cooked ground turkey	0.1–2%	Abuse chilling (54.4 to 7.2°C for 12–21 h)	*Cl. perfringens*	Inhibition of germination	–	Juneja & Friedman (2007)

[a] When no exact concentration could be calculated by the study, description of the experimental procedures is provided.
[b] When no modified atmosphere packaging is reported, the study was performed under aerobic conditions.
[c] The following classification has been used: N, no inhibition (similar increase in logs to the control); L, low inhibition (<1.5 log lower than the control); M, medium inhibition (1.5–2.5 log lower than the control); H, high inhibition (>2.5 log lower than the control).
[d] The following classification has been used: "–", no such response was observed. Log numbers refer to the total reduction compared to the control by the end of storage.
[e] Minimum inhibitory concentration.

1998b). Of the essential oils tested, eugenol and pimento leaf, followed by caraway seed, were the most effective in suppressing the maximum population and/or delaying growth of *A. hydrophila* and to a lesser extent *L. monocytogenes* (Table 10.1), even though the latter showed negligible growth within 14 days at 5°C. The inhibitory effect was more evident at 5°C than at 15°C and at lower initial inoculation levels (10 CFU/g) compared to 10^5 CFU/g. Moreover, more essential oils seemed to be effective in chicken than in beef, especially against *A. hydrophila* inoculated at low cell density. Nadarajah et al. (2005a) reported that 5–20% mustard flour was capable of eliminating 3 log CFU/g of *E. coli* O157:H7 in ground beef packaged under 100% N_2 and stored at 4°C, whereas elimination of 6 logs required at least 20% flour. Bell pepper is another spice with very active EO. In particular, essential oil of bell pepper at concentrations 1.5 ml/100 g and 0.3 ml/100 g of minced meat was capable of completely inhibiting growth of *S. typhimurium* and *Pseudomonas aeruginosa*, respectively, whereas higher concentrations could exert bactericidal effect (Careaga et al., 2003). Moreover, addition of 1% NaCl reduced the required levels of capsicum extract (bell pepper) for inhibition of *P. aeruginosa*. The antimicrobial activity of mustard and horseradish is highly attributed to allyl isothiocyanate (AIT). AIT is considered more effective against Gram-negative bacteria, such as *E. coli* O157:H7 and *Vibrio parahaemolyticus* than Gram-positive bacteria, such as lactic acid bacteria (Ward et al., 1998; Muthukumarasamy, Han, & Holley, 2003; Holley & Patel, 2005).

In order to reduce the binding of EO by food ingredients, and moderate its sensory impact, a promising alternative application is the encapsulation within edible matrices or surfactant micelles. For instance, rosemary oil up to 5% encapsulated in modified starch was more capable of inhibiting *L. monocytogenes* in pork liver sausage than 1% pure EO (Pandit & Shelef, 1994). Encapsulation within surfactant micelles, likely increases the water solubility of hydrophobic EOs, e.g. carvacrol and eugenol, and hence, facilitates their dispersion in the aqueous phase (Gaysinsky, Davidson, Bruce, & Weiss, 2005; Gaysinsky, Taylor, Davidson, Bruce, & Weiss, 2007). Recent studies have also demonstrated that the activities of thyme (Solomakos et al., 2008) or savory and oregano (Ghalfi, Benkerroum, Doguiet, Bensaid, & Thonart, 2007) essential oils in pork and beef may be significantly enhanced, when combined with nisin or bacteriocin-producing lactic acid bacteria (e.g. *L. curvatus*). Moreover, modified atmosphere and lactic acid may also positively contribute to the activity of EOs, e.g. rosemary and clove, against both Gram-negative and Gram-positive members of meat microbial association (Djenane, Sánchez-Escalante, Beltrán, & Roncalés, 2003a, 2003b; Naveena, Muthukumar, Sen, Babji, & Murthy, 2006). In this manner, EOs may also exhibit a protective effect on lipids and/or myoglobin oxidation, and at levels that limit the negative impact on taste and flavour (Chouliara, Karatapanis, Savvaidis, & Kontominas, 2007; Naveena et al., 2006).

The activity of essential oils from sage, clove, rosemary, oregano and thyme is extensively documented in meat and chicken under aerobic, modified

atmosphere packaging (MAP) or vacuum storage (Table 10.1). For instance, rubbed sage at levels up to 2.5% in chicken noodles and strained beef showed considerable inhibition on germination of *B. cereus* spores, no activity was obtained with sage oil up to 5000 ppm (Shelef, Jyothi, & Bulgarelli, 1984). In addition, clove and oregano essential oils possess a wide antimicrobial spectrum, including pathogens and spoilage flora of meat (Table 10.1). Oregano oil not only delayed growth of meat microbial association of ground meat at 5°C, especially Gram-positive flora (e.g. *Brochothrix thermosphacta* and lactic acid bacteria), but also reduced the rate of glucose consumption, the release of a-amino acids and the production of organic acids, compared to the control (Skandamis & Nychas, 2001a). Such effects were more pronounced under 100% CO_2, followed by 40% CO_2/30% O_2/30% N_2 and then aerobic storage, suggesting enhanced activity due to multiple hurdles, namely low temperature, oregano oil and MAP. Moreover, the effectiveness of essential oil increased with concentration from 0.05 to 1% (v/w). Similar effects have been observed with chicken (Chouliara et al., 2007). Levels of oregano oil up to 0.8% also exerted a profound antimicrobial effect against *S. typhimurium* and *L. monocytogenes* and spoilage flora of beef slices under vacuum or MAP, when packaging film of low permeability was used. Limited activity was observed in high permeable pouches, in which MAP and vacuum collapsed during storage (Tsigarida et al., 2000; Skandamis et al., 2000). Furthermore, a beneficial attribute of oregano and thyme oils is their compatibility with the sensory properties of many meat products, such as beef-burgers and smoked sausages. This characteristic renders them promising additives for use in meat preservation. The same may also be the case with garlic, mustard or pepper extracts (El-Khateib & El-Rahman, 1987; Careaga et al., 2003).

Application of Essential Oils in Active Packaging

It is well known that packaging protects foods from microbial or chemical contamination, it ensures mechanical resistance and in some cases, such as vacuum or modified atmosphere packaging, it may delay biological and chemical reactions, thus extending shelf life of packaged foods (Nychas & Skandamis, 2005). As stated above, the combination of MAP or vacuum with EOs may optimize the preservative effect. With the mentioned views, remarkable progress has been made on the development of packaging materials with low permeability in different gases (films of low or high barrier), edible composition and antimicrobial properties. Edible films are coatings made of lipids (e.g. waxes, corn oil, fatty alcohols, etc.), polysaccharides (starch, alginates, carrageenans, cellulose, chitosan) or proteins (e.g. gelatine, collagen, casein, oilseed proteins, etc.) (Gennadios, Hanna, & Kurth, 1997; Nychas & Skandamis, 2005). These films may act as solute, gas and vapour barriers, whereas when antioxidants or antimicrobials are incorporated in the packaging

material, packaging may exert an inhibitory effect on spoilage and pathogenic micro-organisms on the surface of foods (Cutter & Siragusa, 1996; Cutter & Siragusa, 1997; Devlieghere, Vermeiren, & Debevere, 2004; Seydim & Sarikus, 2006; Coma, 2008). An additional innovation on packaging is termed 'smart' or 'active' or 'intelligent' packaging (Davies, 1995). 'Intelligent' or 'smart' packaging can monitor the food and transmit information on its quality, while 'active' can be defined as a type of packaging that changes the condition of the packaging (O_2 scavenging, CO_2 formation, aroma/ethylene/off-flavour/water removal, ethanol emission, release of antimicrobials) to extend shelf life or improve safety or sensory properties while maintaining the quality of the food (Davies, 1995; Vermeiren, Devlieghere, Van Beest, De Kruijf, & Debevere, 1999; Han, 2000; Coma, 2008).

Antimicrobial packaging may be elaborated by the following types of films (Cooksey, 2001): (i) connection of a sachet bearing volatile antimicrobial agents to the inner surface of the packaging material and release of active volatile compounds during storage; (ii) direct incorporation of an antimicrobial agent in the packaging material; (iii) coating of the packaging material with a matrix-carrier, such as chitosan, of the antimicrobial agents; (iv) non-food-grade polymers containing non-diffusible biocides (acting only on the food surface, such as chitosan and triclosan); and (v) edible coatings bearing antimicrobials and applied directly onto the foods as described above (Vermeiren et al., 1999; Aymerich, Picouet, & Monfort, 2008; Coma, 2008; Table 10.2). The antimicrobial agents may either migrate into the food through diffusion and partitioning or be released through evaporation in the headspace. Both latter mechanisms constitute potential applications of EOs, as illustrated in Table 10.2. A primary benefit by including EOs in active packaging is the need for lower concentrations compared to spraying, dipping or direct addition to the food. In this respect, existing reports aim to optimize the antimicrobial effectiveness of EOs in active packaging by selecting the appropriate concentrations of EOs for each food type, considering the fat content and the polar character of the EO, combining packaging with other hurdles, such as irradiation and MAP, as well as by selecting proper films which enable both adhesion of EO on packaging support and high release rate (Gill et al., 2002; Oussalah et al., 2006; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2007; Aymerich et al., 2008; Coma, 2008). Emulsifying agents, such as egg-yolk and lecithin may be used to increase stability of EO within packaging films (Gill et al., 2002; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004). The majority of studies on antimicrobial packaging in relation to EOs have been focused on the application of EO in edible protein-based coatings (Table 10.2). Of the total EOs presented here, AIT and carvacrol seemed to be the most effective (Table 10.2). A general principle, however, is that vapours of EO are less effective than the oil itself (Skandamis et al., 2000; Skandamis & Nychas, 2002; Fisher & Phillips, 2006).

Table 10.2 Use of essential oils or their components in active packaging of foods

Antimicrobial agent	Food	Active packaging system[a]	Experimental conditions	Microorganism	Growth inhibition[b]	Inactivation[c]	References
Horseradish root	Roast beef	Vapours of distillate, diluted in canola oil. **2, 4, 20 µl/l air** on watch glasses inside jars	12°C Inoculum: 10^{2-3} CFU/cm^2	*L. monocytogenes* *S. aureus* *E. coli* O157:H7 *S. typhimurium* *Serratia grimesii* *L. sakei*	*L–M* *L–M* *M–H* *M–H* *H* *N*	–	Ward et al. (1998)
Allyl isothiocyanate (AIT)	Ground beef	Vapours of AIT diluted in corn oil, **1300 ppm** on Whatman inside packaging	4°C under N_2 AIT with 10^3 or 10^6 CFU/g *L. reuteri*	*E. coli* O157:H7 5-strains cocktail 10^3 and 10^6 CFU/g	–	3 to >4.5 log CFU/g	Muthukumarasamy et al. (2003)
Cilantro oil	Ham	Gelatin (7%) – egg yolk coating + **6%** EO (**1000 ppm** on ham)	10°C vacuum Inoculum: 10^4 CFU/cm^2	*L. monocytogenes* (5-strains cocktail)	*L–M*	–	Gill et al. (2002)
Spanish oregano Chinese cinnamon Winter savory	Ham Bologna Ham Bologna Whole beef muscle	Ca (2%, 20%) – alginate edible films with **1%** EO	4°C Inoculum: 10^3 CFU/cm^2	*S. typhimurium* *L. monocytogenes* *S. typhimurium* *E. coli* O157:H7	– – *L* – 	1.5–2 logs 1–1.5 logs – 1.5–3 logs 1–2 logs	Oussalah et al. (2006, 2007)
AIT	Ground beef patties	**0.5, 1 ml** AIT on 10 cm filter paper disc on top of patty	–18, 4, 10°C 100% N_2	Mesophilic counts	*N* at –18 and 10°C *M* at 4°C		Nadarajah et al. (2005b)
				E. coli O157:H7 (5-strains cocktail) 10^3 CFU/g 10^6 CFU/g	– –	2–3 logs –	
Powder of Thyme Rosemary Sage	Ground beef patties	**3% mixture (1:1:1)** in edible film of calcium caseinate and whey proteins	4°C with ascorbic acid and irradiation 1–3 kGy	Natural flora	*N*	–	Ouattara et al. (2002)

Table 10.2 (continued)

Antimicrobial agent	Food	Active packaging system[a]	Experimental conditions	Microorganism	Growth inhibition[b]	Inactivation[c]	References
Cinnamaldehyde	Bologna Cooked ham pastrami	**1%** in chitosan-based film with lauric, acetic or propionic acid	4, 10°C in vacuum Inoculum: 10^4 CFU/cm^2	Natural flora	*H* only on *Enterobacteriaceae*	–	Ouattara et al. (2000)
				L. sakei	*L*	–	
				S. liquefaciens	*H*	–	
Oregano Pimento 1:1 mixture of the two	Whole beef muscle	**1%** diluted in starch-lecithin solutions within edible film of calcium caseinate and whey proteins	4°C Inoculum: 10^3 CFU/g	*Pseudomonas* sp. (3-strains cocktail) *E. coli* O157:H7	*L* only with oregano	–	Oussalah et al. (2004)
Carvacrol	Raw chicken	**20–100%** diluted in ethanol and placed on the lid of Petri dish	4, 20, 37°C for 24 h	*S. enteritidis* Inoculum: 10^3 CFU/g	Total inhibition	–	Burt et al. (2007)
Oregano	Bologna	**1–2%** diluted in Tween 80 within chitosan-based film	4°C	Sensory evaluation	Extension of shelf life	–	Chi et al. (2006)
Oregano	Fresh beef	Filter paper dipped in **100%** oregano for 10 s	5, 15°C – Air – Vacuum – 40%CO_2/ 30%O_2/ 30%N_2 – 80%O_2/ 20% air – 100%CO_2	Natural flora	M in vacuum at 5°C H in CO_2 at 5°C N–L in air and 15°C	–	Skandamis & Nychas (2002)

[a] Reported concentrations are in bold.
[b] The following classification has been used: N, no inhibition (similar increase in logs to the control); L, low inhibition (<1.5 log lower than the control); M, medium inhibition (1.5–2.5 log lower than the control); H, high inhibition (>2.5 log lower than the control).
[c] "–", no such response was observed. Log numbers refer to the total reductions compared to the control by the end of storage.

Chemical GRAS Compounds in Meat and Ready-to-Eat Meat Products

In order to control *L. monocytogenes* in ready-to-eat (RTE) meat and poultry products, which have been identified as foods of high risk for listeriosis according to the draft risk assessment ranking of 23 RTE products (FDA/CFSAN & USDA/FSIS, 2001), the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) undertook two initiatives: (a) the establishment of a final rule (USDA/FSIS, 2003) and (b) the quantitative risk assessment for listeriosis in RTE foods (FDA/CFSAN & USDA/FSIS, 2003). In the first two alternatives, the final rule suggests the use of antimicrobial agents to ensure control of post-process contamination of RTE meat and poultry products. Among the potential chemical antimicrobial agents, certain organic acid salts, especially sodium lactate (SL) and sodium diacetate (SD) (Shelef & Addala, 1994; Shelef, 1994) have received considerable attention, these two are the most commonly used additives as flavour enhancers and shelf life extenders in cured meat products. The permissible levels of SL and SD are 3% (i.e. 4.8% of a commercially available product) and 0.25%, respectively (USDA/FSIS, 2000). The effectiveness of these two compounds on the control of *L. monocytogenes* has been demonstrated, for example, in frankfurters (Bedie et al., 2001; Samelis et al., 2001; Islam, Chen, Doyle, & Chinnan, 2002a; Samelis et al., 2002; Lu, Sebranek, Dickson, Mendonca, & Bailey, 2005; Geornaras et al., 2006a), bologna sausages (Barmpalia et al., 2005; Formato et al., 2007), smoked sausages (Geornaras et al., 2006b), wieners and cooked bratwurst (Glass et al., 2002), chicken luncheon meat (Islam, Chen, Doyle, & Chinnan, 2002b) and cooked ham (Stekelenburg & Kant-Muermans, 2001), each of the above formulated with 0–6% SL and/or 0–0.5% SD or surface treated with 3–6% SL and/or 3–25% SD. However, salts of other organic acids, such as those of sorbic, benzoic and propionic, as well as popular natural antimicrobials, such as nisin have also been reported to control pathogens (Jofré, Garriga, & Aymerich, 2008; Jofré, Aymerich, & Garriga, 2008). A more detailed overview of relevant studies to the activity of chemical GRAS compounds in meat products is presented in Table 10.3. The outcome of these studies may be summarized in the following: (i) in order to achieve the best antimicrobial effect using chemical preservatives below the maximum permissible level, combinations of antimicrobials coupled with refrigeration temperatures are highly suggested; (ii) surface antimicrobial applications require higher amounts of preservatives to get the desired effect than addition of antimicrobials in the formulation of the products; and (iii) treatments involving SD could exert bactericidal effect, whereas SL commonly exhibits bacteriostatic effect.

Risk assessments as well as validation of control strategies require scientific data on both probability of growth and inactivation/growth kinetics of pathogens, and especially *L. monocytogenes*, under storage conditions occurring from production to consumption (i.e. manufacturing, distribution from the establishments in vacuum packages, retail display, as well as storage in household

Table 10.3 Studies on the application of GRAS chemical preservatives in meat and RTE meat products

Food	Antimicrobial agent	Concentration	Application	Conditions	Microorganism, inoculum treatment	Growth inhibition[a]	References
Sausages	Potassium sorbate	0.05%, 1%	Formulation	4°C, single treatments aerobically	*L. monocytogenes*	*a*	Choi & Chin (2003)
	Sodium benzoate	0.05%, 1%				*a*	
	Sodium lactate	3.3%				*a*	
Buffalo minced meat	Sodium acid pyrophosphate	0.5%			*L. monocytogenes*	*a*	Pawar et al. (2002)
	Sodium lactate	2.5%				*a*	
Cooked beef top rounds	Sodium lactate	3%, 4%		4°C, single and combined treatments in vacuum	Aerobic plate counts	*b*	Maca et al. (1997)
	Sodium propionate	0.1%, 0.2%					
Wieners	Sodium lactate	1.32–3.5%	Formulation	4.5°C, single or combined treatments in vacuum	*L. monocytogenes*	*a*	Glass et al. (2002)
	Sodium diacetate	0.1–0.25%					
	Sodium lactate	3–6%	Surface			*a*	
	Sodium diacetate	1.5–3%					
Cooked Bratwurst	Sodium lactate	2–3.4%	Formulation	3, 7°C, single or combined treatments in vacuum		*a*	
	Sodium diacetate	0.1%					
Frankfurters	Sodium benzoate Sodium propionate Potassium sorbate Sodium diacetate	15–25%	Surface	4., 13, 22°C, single treatments aerobically	*L. monocytogenes*	*a*	Islam et al. (2002a)
	Nisin	640 AU/ml	Zein coatings	4°C, single or combined treatments aerobically	*L. monocytogenes*	*b*	Lungu & Johnson (2005)
	Sodium lactate	6%					
	Sodium diacetate	6%					
	Pediocin	3%	Surface	4°C, single or combined treatments in vacuum	*L. monocytogenes*	*b*	Uhart et al. (2004)
	Sodium lactate	6%					
	Sodium diacetate	3%					

Table 10.3 (continued)

Food	Antimicrobial agent	Concentration	Application	Conditions	Microorganism, inoculum treatment	Growth inhibition[a]	References
	Acetic acid Lactic acid, citric acid, tartaric acid, Acetic–Citric	1%, 2% 1%		5°C, in vacuum	*L. monocytogenes*	*a, b*	Palumbo & Williams (1994)
	Sodium lactate, sodium diacetate, potassium sorbate	3 or 6%		–2.2, 1.1, 4.4, 10, 12.8°C, single or combined treatments aerobically	*L. monocytogenes*	*a*	Lu et al. (2005)
	Acetic acid Lactic acid Propionic acid Benzoic acid	2% 1% 0.1% 0.1%		4, 7°C vacuum packaged after steam pasteurization (114°C, 1.5 s)	*L. monocytogenes* *L. innocua*	*b*	Murphy et al. (2006)
	Potassium lactate	2 or 3%	Formulation	4°C, two low initial inoculation levels in vacuum	*L. monocytogenes*	*a*	Porto et al. (2002)
	Sodium diacetate Potassium lactate PURASAL P Optiform 4 (Commercial mixture of potassium lactate-sodium diacetate)	0.1% 3% 2–4%		4°C, single treatments in vacuum	*L. monocytogenes* *L. sakei*	*a* (complete inhibition) *a* (delay in growth)	Stekelenburg (2003)
	Sodium lactate Sodium acetate Sodium diacetate	3, 6% 0.25, 0.5% 0.25, 0.5%			*L. monocytogenes* Natural flora	*a, b* *a*	Bedie et al. (2001)
	Sodium lactate	3%		4°C in vacuum	Lactic acid bacteria	*a*	Drosinos et al. (2006)

Table 10.3 (continued)

Food	Antimicrobial agent	Concentration	Application	Conditions	Microorganism, inoculum treatment	Growth inhibition[a]	References
	Sodium lactate-sodium acetate-potassium sorbate	3–0.5–0.1%					
	Sodium lactate SL-SA SL-SD Glucono-delta-lactone	3% 3–0.25% 0.25%		4°C single or combination treatments, in vacuum, with post-packaging pasteurization (80°C, 60 s)	*L. monocytogenes* Natural flora	*a* *a*	Samelis et al. (2002)
Frankfurters, smoked sausages, ham, bologna	Acetic acid Lactic acid Potassium benzoate Nisin Nisin-AA Nisin-LA Nisin-PB	2.5% 2.5% 5% 0.5% Sequential treatments	Surface	10°C in vacuum, Products with or without 1.5% PL + 0.05% SD in the formulation	Natural flora *L. monocytogenes* Cells previously grown on stainless steel, product purge or laboratory media	*a* *a, b*	Geornaras et al. (2005, 2006a, 2006b)
Bologna	Sodium lactate SL-SD	3% 1.8–0.25%	Formulation	10°C in vacuum	*L. monocytogenes* *Acid- or nonacid adapted*	*a, b*	Formato et al. (2007)
	Sodium lactate Sodium diacetate Glucono-delta lactone	1.8% 0.125% 0.125%		4, 10°C, single or combined treatments in vacuum	*L. monocytogenes* Natural flora	*a*	Barmpalia et al. (2005)
	Acetic acid Lactic acid Sodium lactate Sodium acetate Sodium diacetate	2.5, 5% 2.5, 5% 5, 10% 2.5, 5% 2.5%, 5%	Surface	4°C, single treatments in vacuum	*L. monocytogenes* Natural flora	*a, b*	Samelis et al. (2001)

Table 10.3 (continued)

Food	Antimicrobial agent	Concentration	Application	Conditions	Microorganism, inoculum treatment	Growth inhibition[a]	References
	Potassium benzoate	5%					
	Potassium sorbate	5%					
	Sodium benzoate-sodium propionate	0.05–0.05%	Formulation	4°C in vacuum	*L. monocytogenes*	*a*	Glass et al. (2007)
	Sodium benzoate-potassium sorbate	0.05–0.05%					
	Sodium lactate	2%		5, 10°C in vacuum	*L. monocytogenes*	*a*	Qvist et al. (1994)
	Glucono-delta lactone	0.25%, 0.5%					
Ham	Potassium lactate-sodium diacetate	Not specified	Formulation	4°C, simulation of contamination at retail level	*L. monocytogenes*	*a*	Lianou et al. (2007)
	Nisin Potassium lactate	800 AU/g 1.8%		1, 6°C, single or combined treatments with ultra high pressure (600 MPa)	*L. monocytogenes* *Salmonella* sp. *Staph. aureus*	*a, b*	Jofré et al. (2008)
	Enterocins A, B	200, 2000 AU/cm^2	Antimicrobial packaging (Polypropylene-polyamide)	6°C, in vacuum with UHP (400 MPa)	*Salmonella* sp.	*b*	Jofré et al. (2008b)
	Sakakin K	200, 2000 AU/cm^2					
	Nisin	200 AU/cm^2					
	Potassium lactate	1.8%					
	Nisin-PL						
	Enterocins	2400 AU/cm^2	Formulation	1, 6°C in vacuum with UHP (400 MPa)	*L. monocytogenes*	*a, b*	Marcos et al. (2008)
	Potassium lactate-sodium diacetate	1.4–0.1% 2.5% (PURASAL Opti form 4)					

Table 10.3 (continued)

Food	Antimicrobial agent	Concentration	Application	Conditions	Microorganism, inoculum treatment	Growth inhibition[a]	References
	Sodium lactate Sodium diacetate Sodium citrate	2.5%, 3.3% 0.1%, 0.2% 1%		4°C in vacuum	*L. monocytogenes* *L. curvatus*	*a*	Stekelnburg & Kant-Muermans (2001)
	Commercial mixtures (PURASAL) of potassium lactate–sodium diacetate	3% of 54–57% PL + 3.7–4.3% SD or 3% of 58–62% PL		4, 8°C in vacuum	*L. monocytogenes*	*a*	Mellefont & Ross, (2007)
Ham, turkey	Sodium lactate Sodium diacetate Sodium benzoate Potassium sorbate Sodium propionate	1.6% 0.1% Up to 0.3%		4°C in vacuum	*L. monocytogenes*	*b*	Glass et al. (2007)
Ham, servelat sausage	Sodium lactate Sodium acetate	2.5% 0.25%		4, 9°C in vacuum	*L. monocytogenes* Lactic acid bacteria	*a*	Blom et al. (1997)
Cook-in-bag turkey	Potassium lactate–sodium diacetate	1.54–0.11%	Formulation	4°C, dipping into 15 ml of acidified sodium chlorite (250 ppm) and pasteurized (95°C, 3 min). Vacuum packaging	*L. monocytogenes*	*a*	Luchansky et al. (2006)
Beef strip stakes	Sodium lactate Sodium lactate–sodium diacetate	30% 30–2.5%	Niddle injection	4°C, in vacuum	*E. coli*	*a*	Wicklund et al. (2007)

Table 10.3 (continued)

Food	Antimicrobial agent	Concentration	Application	Conditions	Microorganism, inoculum treatment	Growth inhibition[a]	References
Meat slurries, comminuted meat, model meat systems	Sodium lactate Sodium diacetate Sodium acetate	1.8, 2.5% 0.1, 0.2% 0.2%	Formulation	5, 10°C, single or combined treatments, aerobically	*S. enteritidis*	*a, b*	Mbandi & Shelef (2001)
	Sodium diacetate Sodium lactate Sodium diacetate Pediocin	0.1–0.5% 2.5% 0.1–0.5% 5000 AU/ml		4, 25°C, single or combined with commercial antimicrobials aerobically or in vacuum	*L. monocytogenes*	*a, b* *a, b*	Schlyter et al. (1993a, b)
	Sodium lactate	2–4%		10°C, in vacuum	*L. monocytogenes* *S. aureus* *S. typhimurium* *E. coli* O157:H7 *Cl. perfringens*	*a* *a* *a* *a* *a*	Miller & Acuff (1994)
Chicken luncheon meat	Sodium benzoate, sodium propionate, potassium sorbate, sodium diacetate	15–25% (up to 0.15% on surface)	Surface (Spraying)	4, 13, 22°C, single treatments aerobically	*L. monocytogenes*	*b*	Islam et al. (2002b)
Fresh beef	Polylactic Lactic acid Nisin	2% 2% 200 IU/ml	Surface	4°C, single treatments in vacuum	Natural flora	*a*	Ariyapitipun et al. (1999)
Ham, bologna	Lysozyme Nisin	6.06 g/l 430 mg/l	Gelatin coating	8°C with EDTA, in vacuum	*E. coli* O157:H7 *S. typhimurium* *L. monocytogenes*	*b*	Gill & Holley (2000)

[a] *a* – bacteriostatic effect, *b* – bactericidal effect.

refrigerators). In this respect, predictive modelling may serve as a useful tool to quantitatively express the effectiveness of control measures, including chemical preservatives, on the behaviour of *L. monocytogenes*. Given the recent need for use of chemical agents to increase the safety of RTE products, predictive models have been developed primarily to express the inactivation of *L. monocytogenes* in cured meat products as a function of heating temperature (55–73.9°C), sodium lactate (0–4.8%) and sodium diacetate (0–0.25%), sodium pyrophosphate, (0–0.5%) and NaCl (0–6%) (Juneja, 2003; Lihono, Mendonca, Dickson, & Dixon, 2003; Schultze, Linton, Cousin, Luchansky, & Tamplin, 2006). Such models aim to simulate the log reduction that may be achieved during cooking of products formulated with the above preservatives. Predictive models are also available for the combined effect of SL (in mM) with temperature (4–30°C) and pH (5.5–7.0) (Houtsma, Kusters, De Wit, Rombouts, & Zwietering, 1994; Houtsma, Kant-Muermans, Rombouts, & Zwietering, 1996), or the combined effect of SL (3 and 6%), SD (3 and 6%) and temperature (1.1–12.8°C) (Lu et al., 2005), or the combined effect of potassium lactate (PL; 0.25–9.25%), SD (0–0.25%) and NaCl (0.8–3.60%) (Seman, Borger, Meyer, Hall, & Milkowski, 2002) on the growth of *L. monocytogenes* (or *L. innocua*) on a variety of cured or uncured meat and poultry products. Development of these models aimed to assist in the determination of the proper amounts of SL or PL and SD to completely inhibit growth of *L. monocytogenes* in RTE meat and poultry products, mainly those packaged under vacuum.

The contribution of models for the probability of growth initiation of *L. monocytogenes* in response to the above variables is also significant in reducing the uncertainty and variability of risk assessments. Thus, preliminary studies have been performed to determine the minimum inhibitory concentration of SL and SD against a variety of spoilage and pathogenic micro-organisms, alone or in combination with other ecological factors, such as pH and temperature. In particular, Houtsma, De Wit, & Rombouts, (1996) showed that the minimum inhibitory concentration (MIC) of SL is strongly pH dependent, since at pH close to 7 SL is inhibitory as a humectant, while at lower pH, the organic anion of SL exerts further antimicrobial activity. Recently, Legan, Seman, Milkowski, Hirschey, and Vandeven (2004) modelled the growth boundaries of *L. monocytogenes* in nine different vacuum packaged RTE cooked meat products formulated with various levels of PL, SD and NaCl at one temperature (4°C). Furthermore, Skandamis, Stopforth, Yoon, Kendall, and Sofos (2007) modelled the combined effect of SL (0–6%) and SD (0–0.5%) in the presence of 0.5 or 2.5% NaCl on the probability of *L. monocytogenes* growth under aerobic and anaerobic conditions. Anaerobic conditions seemed to enhance the antimicrobial effect of SL and SD compared to incubation under aerobic conditions.

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Chapter 11
Biopreservation

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Introduction

In the last decades important changes have been observed in the food science area, with increasing consumers demand for ready-to-eat (RTE) and minimally processed foods, as a reflection of the increasing awareness of the risks derived not only from foodborne pathogens but also from artificial chemical preservatives used to control them (Castellano, Belfiore, Fadda, & Vignolo, 2008; Parada, Caron, Medeiros, & Soccol, 2007; Rodríguez, Martínez, Horn, & Dodd, 2003; Schuenzel & Harrison, 2002). This tendency allied to strict government requirements for food safety has faced food producers with conflicting challenges (Settanni & Corsetti, 2008). The preservation techniques used in early days relied, without any understanding of the microbiology, on the inactivation of undesirable microorganisms through drying, salting, heating, or fermentation. These methods are still used today, combining various hurdles to inhibit growth of microorganisms, but some of the classic preservation techniques are not suitable for fresh meats and RTE products (Gram et al., 2002; Quintavalla & Vicini, 2002; Rao, Chander, & Sharma, 2008).

Meat is a nutrient-rich matrix that provides a suitable environment for proliferation of many spoilage microorganisms and foodborne pathogens (Ananou, Garriga, et al., 2005; Aymerich, Picouet, & Monfort, 2008; Hugas, 1998). Microbial contamination of meats has been implicated with the most serious foodborne outbreaks and recalls from the food marketplace (Ananou, Garriga, et al., 2005; Sofos, 2008). Major causes of concern and product recalls associated with fresh meat products are *Escherichia coli* O157:H7 and related enteric pathogens such as *Salmonella*, while the Gram-positive *Listeria monocytogenes* is the pathogen of concern in RTE meat and poultry products. *L. monocytogenes* is a psychrotrophic bacterium that can grow during

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refrigerated storage of meats, even after a lethality treatment, if recontamination occurs during slicing, packaging, peeling, or handling (Sofos, 2008; Trivedi, Reynolds, & Chen, 2008).

To reduce the level of microbial contamination on raw meats and animal carcasses, processing facilities of all sizes in the United States are currently required to establish Sanitation Standard Operating Procedures (SSOP) as well as the Hazard Analysis and Critical Control Points (HACCP) program (Aymerich et al., 2008; Trivedi et al., 2008). Several carcass decontamination methods have been validated for use as critical control points to reduce bacterial populations on meat and poultry, including steam/hot water vacuuming, spray washing, and steam pasteurization (Trivedi et al., 2008).

The need for alternatives to extend the shelf life of foods without changing their sensory properties has launched research on biopreservation technologies, which are based on the use of non-pathogenic microorganisms and/or their metabolites to retard food spoilage and/or to improve food safety (De Martinis, Públio, Santarosa, & Freitas, 2001; Ross, Morgan, & Hill, 2002).

Methods of Biopreservation

Some pathogens present in foods may be inhibited or even eliminated by the action of competitors or antagonistic microbiota, improving the shelf life and safety of products without the need of using elevated levels of chemical additives (Schuenzel & Harrison, 2002). The presence of a competitive microbiota is a promising alternative also to prevent biofilm formation by some pathogens in food processing equipments (Jeong & Frank, 1994; Minei, Gomes, Ratti, D'Angelis, & De Martinis, 2008).

Lactic acid bacteria (LAB) have a major potential for use in biopreservation because they have a long history of safe consumption and they naturally dominate the microflora of many foods during storage. Moreover, in raw meats and fish that are chill stored under vacuum or under elevated CO_2 concentration, LAB become the dominant population and preserve the meat with a "hidden" fermentation (Stiles, 1996). The same rationale applies to processed meats if LAB survive heat treatment or if they are reintroduced in the product after heat treatment (Stiles, 1996).

Biopreservation by Lactic Acid Bacteria

LAB constitute a group of Gram-positive, catalase-negative cocci or rods with similar characteristics and the ability to produce lactic acid as the main product of the fermentation of carbohydrates. Many of these microorganisms are considered "food grade" and may exert their antimicrobial properties against pathogens, spoilage bacteria, yeasts, and molds by different ways such as

(a) production of volatile acids, hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl, acetaldehyde; (b) competitive exclusion; and (c) production of bacteriocins (Naidu, Bidlack, & Clemens, 1999; Settanni & Corsetti, 2008). Bacteriocins are antimicrobial peptides produced by numerous Gram-positive and Gram-negative organisms, but bacteriocins from LAB are of special interest in the food science area because LAB present a positive association with foods and have a long history of safe consumption, as part of the natural microbiota of meat, milk, vegetable and fish products (Rodríguez et al., 2003; Aymerich et al., 2008). Moreover, the use of LAB and/or their metabolites for food preservation is generally accepted by consumers as something "natural" and "health promoting" (Deegan, Cotter, Hill, & Ross, 2006; Rodríguez et al., 2003). Bacteriocins of LAB present potential applications in meats, as shown in Table 11.1.

Bacteriocins can be applied in meat systems by two basic methods: by adding crude, purified, or semi-purified bacteriocin preparations or by inoculation with pure cultures of the bacteriocinogenic strains. Both approaches offer advantages and disadvantages, and the choice of either one will depend on the bacteriocin, the producer strain, the food system, and the target microorganism (Ananou, Maqueda, Martínez-Bueno, Gálvez, & Valdivia, 2005; Hugas, 1998). In meat environments, a higher concentration of bacteriocin-producing cells may be necessary to compensate adsorption of bacteriocin molecules to the meat matrix (Ananou, Garriga, et al., 2005).

Before using a given bacteriocin for biopreservation, it is necessary to study its efficacy for each particular food system, to determine the concentrations of bacteriocin required to achieve an efficient control of foodborne pathogens, or the capacity of bacteriocinogenic strains for growth and bacteriocin production in the food system (Ananou, Garriga, et al., 2005).

The naturally occurring LAB strains in meat and meat products include *Carnobacterium piscicola* and *C. divergens*; *Lactobacillus sakei*, *Lb. curvatus*, and *Lb. plantarum*; *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lc. gelidum*, and *Lc. carnosum*. LAB play an important role in fermented foods, causing flavor and texture changes together with a preservative effect resulting in products with increased shelf life (Hugas, 1998; Settanni & Corsetti, 2008).

LAB such as *Carnobacterium* spp., *Lactobacillus* spp., and *Leuconostoc* spp. are those related to meat spoilage, but a selective promotion of growth of LAB capitalizing on their ability to control meatborne pathogens with a preferential growth of benign strains would minimize detrimental effects (Settanni & Corsetti, 2008).

In fermented meat products, *Enterococcus* spp., especially *E. faecium*, represents one of the LAB species that can be found in relatively high numbers during fermentation and they may contribute to the flavor of products by their glycolytic, proteolytic, and lipolytic activities (Ananou, Garriga, et al., 2005). Bacteriocin-producing enterococci are widespread in nature and strains with strong anti-listerial activity have been isolated from numerous fermented meat products and have been well characterized (Belgacem, Ferchichi, Prévost, Dousset, & Manai, 2008).

Table 11.1 Some examples of microorganisms that may be controlled by biopreservation techniques

Target microorganism	Meat products	Biopreservative agent	References
Listeria monocytogenes	Package ground beef and pork steak	Bacteriocin 32Y from *Lactobacillus curvatus*	Ercolini et al. (2006); Mauriello et al. (2004)
Total aerobic bacteria	Frankfurters and fresh veal meat	Nisin	Guerra et al. (2005)
L. monocytogenes	Ready-to-eat turkey bologna	Nisin plus lysozyme	Mangalassary et al. (2007)
Lb. sakei and Lb. curvatus	Bologna-type sausage	Nisin	Davies et al. (1999)
Clostridium perfringens, Salmonella Kentucky, and *L. innocua*	Fresh pork sausage	Lacticin 3147 from *Lactococcus lactis* DPC 3147	Scannell et al. (2000)
L. monocytogenes	Brazilian sausage	*Lb. sakei* 2a bacteriocin producer	De Martinis and Franco (1998)
L. monocytogenes	Fish peptone model system	*Carnobacterium piscicola*	Alves, De Martinis, Destro, Fonnesbech, and Gram (2005)
L. monocytogenes	Cooked ham	*Lb. sakei* 1 bacteriocin producer	Alves, Martinez, Lavrador, and De Martinis (2006)
L. monocytogenes	Meat gravy system	*Lb. sakei* 1 bacteriocin producer	Alves, Lavrador, and De Martinis (2003)
L. monocytogenes	Cooked ham	Enterocins A and B, sakacin K and nisin plus lactate	Jofré et al. (2007)
L. monocytogenes, Brochothrix thermosphacta, Campylobacter jejuni and Cl. estertheticum	Vacuum-packed chill-stored meat	*Lb. sakei* and *Lact. lactis*	Jones, Hussein, Zagorec, Brightwell, and Tagg (2008)
L. monocytogenes and Escherichia coli O157:H7	Dry sausage	Lactic acid bacteria	Työppönen, Petäjä, and Mattila-Sandholm (2003)
L. monocytogenes	Meat	Lactic acid bacteria	De Martinis et al. (2001)
Total plate count	Brined shrimp	Nisin Z, camocin UI49 from *Cb. piscicola* and bavaracin A from *Lb. bavaricus* MI 401	Einarsson and Lauzon (1995)

Bacteriocins of Lactic Acid Bacteria

Bacteriocins produced by LAB may be very attractive for biopreservation due to (i) production by strains generally recognized as safe, (ii) lack of action against eukaryotic cells, (iii) inactivation by digestive proteases, which preserve the gut microbiota, showing no cross-resistance with antibiotics, (iv) tolerance to pH and heat, (v) mostly bactericidal mode of action, and (vi) genetic

determinants usually plasmid encoded, facilitating genetic manipulations (Gálvez, Abriouel, López, & Omar, 2007).

However, many bacteriocins have not been fully characterized and, consequently, cannot be extensively used in the food industry. To date, the only bacteriocin licensed as a food preservative is nisin (Cleveland, Montville, Nes, & Chikindas, 2001; Gálvez et al., 2007; Sobrino-López & Martín-Belloso, 2008). This antimicrobial peptide is produced by *Lactococcus lactis* subsp. *lactis* and marketed under the trade name Nisaplin, which contains ca. 2.5% of nisin (product description PD45003-7EN; Danisco, Copenhagen, Denmark). Alternatively, strains producing bacteriocins can be added to concentrates originating from a food-grade substrate (milk or whey), with commercial applications such as pediocin PA-1 produced by *Pediococcus acidilactici* commercialized as ALTA™ 2341 (Kerry Bioscience, Carrigaline, Co. Cork, Ireland) (Deegan et al., 2006; Gálvez et al., 2007; Sobrino-López & Martín-Belloso, 2008). Commercial bioprotective cultures (Chr. Hansen, Denmark) have been developed to reduce the incidence of spoilage microbiota and some pathogens during processing of meat products: SafePro® B-2 (containing non-bacteriocin-producing *Lb. sakei* BJ33) and SafePro® B-SF-43 (containing bacteriocin-producing *Lc. carnosum* 4010). Also, several patents have been deposited dealing with biopreservation (Cleveland et al., 2001).

Pediocin PA-1 is a plasmid-encoded class II bacteriocin with a broad inhibitory spectrum against *E. faecalis*, *Staphylococcus aureus*, *Clostridium perfringens* and particularly effective against *L. monocytogenes* (Guerra, Bernárdez, & Castro, 2007; Reviriego et al., 2005; Sobrino-López & Martín-Belloso, 2008).

Although pediocin PA-1 is mainly used in vegetables and meat products, the extension of its application to dairy products is being evaluated due to its antilisterial activity, stability in aqueous solutions, wide pH range for activity, and the fact that it is unaffected by heating or freezing (Sobrino-López & Martín-Belloso, 2008; Reviriego et al., 2005).

Nisin was discovered in 1928, after observations that certain lactococcal strains inhibited other LAB in dairy fermentations, and it is currently approved for use in over 50 countries (McAuliffe, Ross, & Hill, 2001; Ross et al., 2002; Delves-Broughton, 2005; Deegan et al., 2006). This bacteriocin is effective in a number of food systems, inhibiting the growth of a wide range of Gram-positive bacteria and their spores, but it does not inhibit the growth of yeasts and molds (Deegan et al., 2006).

Stevens, Sheldon, Klapes, and Klaenhammer (1991) hypothesized that the cell wall of Gram-negative bacteria, composed of lipopolysaccharides, acts as a permeability barrier, preventing nisin from reaching the target cytoplasmic membrane. Those authors affirmed that chelators, hydrostatic pressure, or cell injury may destroy the cell wall, rendering the Gram-negative bacteria sensitive to the bacteriocin. However, the application of nisin to meats may be limited due to its low solubility in meat pH, the inability of the producer organism to grow in meats, and to its inefficiency to inhibit all the spoilage and pathogenic microorganisms associated with meats (Stiles & Hastings, 1991).

Moreover, Rose, Palcic, Sporns, and Mc Mullen (2000) demonstrated that nisin may be inactivated by the enzyme glutathione S-transferase of raw beef, confirming that the use of this bacteriocin in raw meats may be limited. Nisin use for partial replacement of nitrite in cured meats has been investigated and only high and uneconomic levels of nisin may promote good control of *C. botulinum* (Delves-Broughton, 2005). However, better results have been achieved for the use of nisin to overcome post-processing contamination of meat products where LAB can cause spoilage (Aymerich et al., 2008; Delves-Broughton, 2005). The application of nisin in vacuum-packed cooked sausage has achieved regulatory approval in the United States (Delves-Broughton, 2005). In Brazil, nisin was approved for use in cheeses and also for spraying on the surface of frankfurters at the end of the thermal processing step. In fresh meat, nisin has also been tested as spray to sanitize the surface of red meat carcasses (Aymerich et al., 2008).

The use of nisin in meats is still controversial, although it has been reported to present better action in products with lower fat levels (Castro, 2002; Davies et al., 1999; Delves-Broughton, 2005; El-Katheib, Yousef, & Ockerman, 1993; Fang & Lin, 1994)

It has been postulated that bacteriocins and/or protective cultures may be more effective if used in the hurdle technology approach, in combination with other barriers such as modified atmosphere packaging, hydrostatic pressure, high temperature, chelating agents, antimicrobials, and lactoperoxidase system (Chen & Hoover, 2003; Cleveland et al., 2001).

According to Garcia, Martin, Sanz, and Hernández (1995) and Mc Mullen and Stiles (1996) the most suitable strains to be used in biopreservation of a certain food product are likely those isolated from the same type of food where they are intended to be used. They attributed this probability to competitive advantage of the previously adapted strains. Based on this premise, several studies for the isolation of bacteriocinogenic LAB from meats have been conducted in several countries (De Martinis, Alves, & Franco, 2002).

Some bacteriocins presenting anti-listerial activity in meat homogenates have been applied experimentally as ingredients in several meat products, such as enterocin A, enterocin B, and sakacin K (Jofré, Garriga, & Aymerich, 2007).

Enterocin AS-48, produced by *E. faecalis* S-48, exhibits bactericidal activity against a wide variety of Gram-positive bacteria, including food spoilage and pathogenic bacteria such as *Bacillus cereus*, *C. botulinum*, *C. difficile*, *C. perfringens*, *S. aureus*, and *L. monocytogenes*. It also shows activity against some Gram-negative species (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003; Ananou, Garriga, et al., 2005; Lucas et al., 2006). Some features of AS-48 such as (i) broad spectrum of antimicrobial activity, (ii) stability in a wide range of temperature and pH, and (iii) sensitivity to digestive proteases render this bacteriocin a promising alternative to chemical preservatives (Ananou, Garriga, et al., 2005; Ananou, Maqueda, et al., 2005, Lucas et al., 2006).

Recently, a database containing calculated or predicted physicochemical properties of diverse bacteriocins was created and named BACTIBASE (http://bactibase.pfba-lab.org), which can be an efficient tool to facilitate future food biopreservation studies (Hammami, Zouhir, Hamida, & Fliss, 2007). Besides, the elucidation of the mode of action of these antimicrobial peptides can help to optimize their food applications.

Mode of Action of Bacteriocins

The family of bacteriocins includes a diversity of proteins in terms of size, microbial targets, mode of action, and immunity mechanism (Riley & Wertz, 2002). Bacteriocins are proteins ribosomally synthesized and are often confused in literature with antibiotics (Cleveland et al., 2001). They differ from antibiotics because they have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producer strain (Riley & Wertz, 2002). Other differences are that antibiotics are generally considered secondary metabolites and are not ribosomally synthesized (Cleveland et al., 2001; Deegan et al., 2006).

As a group, bacteriocins act on target cells by various mechanisms: (i) permeabilization of the cytoplasmic membrane followed by leakage of low-molecular-weight cellular compounds and dissipation of the proton motive force (PMF); (ii) cell lysis; (iii) degradation of vital macromolecules such as DNA and RNA; and (iv) inhibition of biological processes such as synthesis of protein, DNA, RNA, and peptidoglycan (De Martinis et al., 2002; Motta, Flores, Souto, & Brandell, 2008).

Bacteriocins were first characterized in Gram-negative bacteria (Cleveland et al., 2001). Colicins of *E. coli* are well studied and can act as membrane-depolarizing agents, DNA or RNA endonucleases, translation blocker, or inhibition of murein synthesis (Cursino, Smarda, Chartone-Souza, & Nascimento, 2002; Kolade et al., 2002).

Bacteriocins may be classified into four classes based on their biochemical and genetic properties (Deegan et al., 2006; Drider, Fimland, Héchard, McMullen, & Prévost, 2006; Naghmouchi, Kheadr, Lacroix, & Fliss, 2007). Class I peptides are the lantibiotics, which are small and characterized by unusual amino acids, such as lanthionine, and nisin is included in this class. Class I is subdivided into class Ia and Ib. In general, class Ia bacteriocins consist of cationic and hydrophobic peptides and class Ib bacteriocins are globular peptides with no net charge or a net negative charge (Cleveland et al., 2001). Class II comprise small heat-stable, non-modified peptides and are subdivided into three subclasses, namely, class IIa (pediocin-like bacteriocins), IIb (two different peptides), and IIc (one-peptide bacteriocins). The class III peptides are large and thermosensitive (Cleveland et al., 2001; Drider et al., 2006; Oppegard, Rogne, Emanuelsen, Kristiansen, Fimland, & Nissen-Meyer, 2007). A fourth class contains complex bacteriocins

that are composed of protein plus one or more chemical moieties (lipid, carbohydrate) required for activity: plantaricin S, leuconocin S, lactocin 27, pediocin SJ-1 (De Martinis et al., 2002). Class IV is currently the subject of discussion and not formally recognized since this class has not been studied sufficiently at the biochemical level. Studies have principally focused on members of class I and class II due to the abundance of these peptides and their potential for commercial applications (McAuliffe et al., 2001; Naghmouchi, Drider, & Fliss, 2007).

According to Parisien, Allain, Zhang, Mandeville, and Lan (2008) lantibiotics inhibit target cells by forming pores in the membrane, depleting the transmembrane potential and/or the pH gradient, resulting in the leakage of cellular materials (Cleveland et al., 2001; Deegan et al., 2006; McAuliffe et al., 2001). The electrostatic interactions between the positive charge of bacteriocins and the negative charge of phosphate groups on target cell membranes are thought to contribute to the initial binding with the target membrane. It is likely that the hydrophobic portion inserts into the membrane, forming pores (Cleveland et al., 2001; Deegan et al., 2006). There are two models for pore forming, the barrel stave and the wedge. In barrel stave model, each nisin molecule orients itself perpendicular to the membrane forming an ion channel that spans the membrane (Fig. 11.1A). According to the wedge model, after a critical number of nisin molecules associate with membrane, they insert concurrently, forming a wedge (Fig. 11.1B, C) (Cleveland et al., 2001; McAuliffe et al., 2001; Bauer & Dicks, 2005).

Besides pore formation, it is believed that nisin also mediates inhibition of cell wall biosynthesis, by forming a complex with lipid II, the bactoprenol-bound peptidoglycan precursor (Deegan et al., 2006; Héchard & Sahl, 2002; McAuliffe et al., 2001). It suggests that nisin may use lipid II as a docking molecule for facilitating the interaction with the bacteriocin and specific membranes (Fig. 11.2) (Cleveland et al., 2001). The combination of two killing mechanisms, inhibition of the peptidoglycan synthesis and the pore formation, renders nisin active at nanomolar concentrations (Deegan et al., 2006; Héchard & Sahl, 2002).

Class II bacteriocins predominantly act by inducing permeabilization of the target cell membrane, probably by forming ion-selective pores which cause dissipation of the proton motive force, depletion of intracellular ATP, and leakage of amino acids and ions (Deegan et al., 2006; Drider et al., 2006).

Class IIa is the largest and most extensively studied subgroup of class II bacteriocins that are especially strong inhibitors of *L. monocytogenes*. Because of this anti-listerial effectiveness class IIa bacteriocins have significant potential as biopreservatives in a large number of foods (Ennahar, Sashihara, Sonomoto, & Ishizaki, 2000). Pediocin PA-I and other identical bacteriocins produced by *P. acidilactici* (pediocin AcH, pediocin SJ-I, pediocin JD) are the most extensively studied class II bacteriocins. This bacteriocin was found to induce the leakage of K^+, amino acids, and other low-molecular-weight molecules, which lead to rapid depletion of intracellular bacterial ATP (Drider et al., 2006). Pediocin PA-I dissipates the membrane potential and causes release of amino acids accumulated

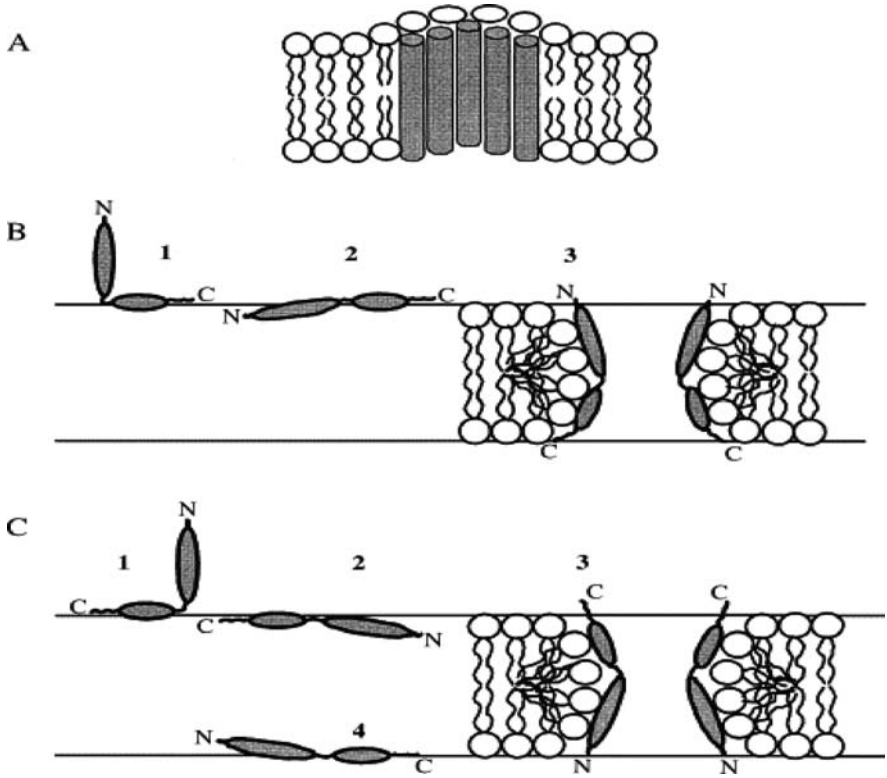

Fig. 11.1 Models of non-targeted pore formation by nisin. (**A**) Barrel stave pore. (**B**, **C**) General models for pore formation. Step 1: binding of nisin via its C-terminal. Step 2: insertion of nisin into the membrane. The depth of insertion depends on the percentage of anionic lipids and nisin concentration. Step 3: wedge/magainin-like pore. Diagrams **B** and **C** represent pore formation initiated by translocation of the C-terminus and N-terminus, respectively. Step 4: translocation of the peptide to the inside of the membrane (Bauer & Dicks, 2005)

either in a proton motive force (PMF)-dependent or -independent manner (Héchard & Sahl, 2002).

There is less information about bacteriocin action mechanism against spores than there is for vegetative cells. Most of these studies deal with nisin, which is sporostatic rather than sporocidal. It was found that nisin modifies the sulfhydryl groups in the envelopes of germinative spores, acting as electron acceptors (Montville, Winkowski, & Ludescher, 1995).

Several factors influence the bacteriocin activity on the target bacterial cell (Héchard & Sahl, 2002; Motta et al., 2008). These include the structure and amount of the substance, the composition of the cytoplasmic membrane, the structure and the expression level of a protein with an immunity function, and the chemical composition of the environment (Ennahar et al., 2000; Héchard &

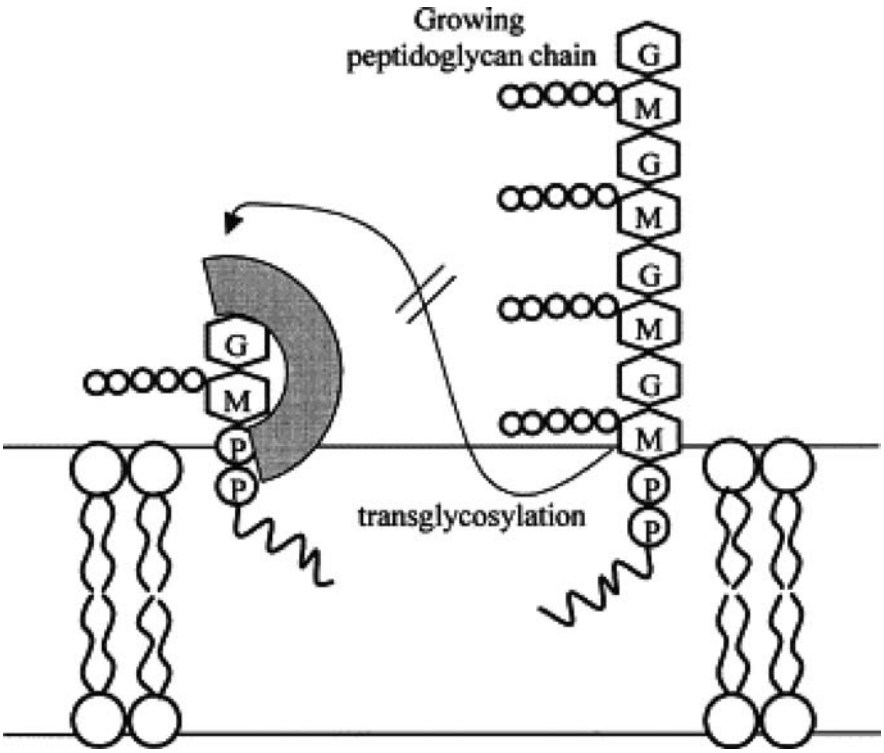

Fig. 11.2 Model for lipid II-mediated inhibition of peptidoglycan biosynthesis. Lantibiotics (marked by *shading*) such as nisin and the mersacidin subtype bind to lipid II, thereby blocking the polymerization of the peptidoglycan. The recognition site for nisin is MurNAc, whereas mersacidin interacts with GlcNAc. Interaction with the pyrophosphate (PP) moiety of lipid II may be involved in stabilizing the transmembrane orientation of the peptides (Bauer & Dicks, 2005)

Sahl, 2002). Thus, the effective use of bacteriocins in food preservation requires the understanding of their mode of action and inhibitory action under different biochemical conditions naturally occurring in foods (Motta et al., 2008).

There is concern on the development of resistance to bacteriocins and also that exposure to bacteriocins renders target microbial cells more resistant to antibiotics (Martínez, Obeso, Rodríguez, & García, 2008). Genetically stable bacteriocin-resistant organisms have been generated with a frequency of 1 in 10^6 cells under optimal growth conditions (Harris, Fleming, & Klaenhammer, 1991; Ming & Daeschel, 1993). Nisin-resistant cells have already been observed for *L. monocytogenes*, *S. aureus*, *C. botulinum*, and *B. cereus*. However, no cross-resistance to antibiotics has been observed, likely due to different modes of action of bacteriocins and antibiotics (Cleveland et al., 2001). It has been shown that bacteriocin resistance results from physiological changes in target cell membrane or production of an enzyme that degrades bacteriocin, while

antibiotic resistance is generally associated with genetic determinants. Cross-resistance between different class IIa bacteriocins has been reported and it seems to be related to changes in phosphotransferase systems (PTSs), responsible for the uptake and concomitant phosphorylation of a number of sugars in bacteria (Gravesen et al., 2002). Moreover, according to Gravesen et al. (2002) since food systems are inherently heterogeneous, many interacting factors will influence the development of bacteriocin resistance and need to be further investigated.

Future Perspectives

The application of bacteriocins as food additives demands an exhaustive evaluation. Before being legally accepted, their use and efficacy must be shown and they must be chemically identified and characterized. Moreover, manufacturing process and assays used for quantification and standardization of peptide must be described; in addition, toxicological data and fate of molecule after ingestion are also needed (Cleveland et al., 2001; Sobrino-López & Martín-Belloso, 2008). The potential applications of bacteriocins from LAB in the food and health care sectors are evident. However, for effective commercial application and for production in large scale, both genetic and fermentative protocols need to be optimized (Guerra, Agrasar, Macías, Bernárdez, & Castro, 2007; Kim & Mills, 2007).

Nowadays researchers are focusing on the application of bacteriocins in foods as part of packaging films, since microbial contamination of meat products occurs primarily at the surface, due to post-processing handling (Coma, 2008). One strategy for reducing contamination is to entrap the antimicrobials in an edible film matrix packaging, which allows a slow migration to the food surface and helps to maintain high concentrations of the biopreservative as needed (Cagri, Ustunol, & Ryser, 2004). Antimicrobial packaging films have been studied to deliver bacteriocins as an additional barrier to control microbial growth (Cagri et al., 2004; Cha & Chinnan, 2004; Ercolini, Storia, Villani, & Mauriello, 2006; Guerra, Macias, Agrasar, & Castro, 2005; Mauriello, Ercolini, La Storia, Casaburi, & Villani, 2004; Ming, Weber, Ayres, & Sandine, 1997; Quintavalla & Vicini, 2002; Siragura, Cutter, & Willet, 1999).

Recent trends in bacteriocin research also involve heterologous production of LAB bacteriocins to construct multi-bacteriocinogenic strains or to confer antimicrobial properties to strains of technological interest, such as those used as starter cultures (Rodríguez et al., 2003). Cloning and expression of bacteriocin genes in new hosts have allowed to constitute production and even overexpression of bacteriocins, therefore overcoming bacteriocin regulation systems (Ennahar et al., 2000).

E. coli has long been considered the primary prokaryotic host for cloning and expressing heterologous genes due to its extensive genetic characterization (Billman-Jacobe, 1996). Consequently, this bacterium has invariably been

selected as the first host for cloning a variety of genes involved in the biosynthesis of many LAB bacteriocins, but alternative food-grade organisms must be employed when production of recombinant proteins in industrial food products is desired. Many LAB species or strains are potentially useful for the heterologous production of commercially important proteins or peptides since they fulfill this requirement of food-grade organisms (Rodríguez et al., 2003).

As an example, pediocin PA-1-producing bacteria are pediococci, usually associated with vegetables and meat products but not suitable for production of dairy products. Since this species is unable to ferment lactose, it is metabolically and technologically unsuitable for dairy fermentation. Attempts have been made to achieve the heterologous expression of pediocin PA-1 in *Lact. lactis* or of acidocin A in *Lb. casei* for production of the bacteriocin during the lactic fermentation process (Reviriego, Fernández, Kuipers, Kok, & Rodríguez, 2007; Reviriego, Fernández, & Rodríguez, 2007). Heterologous expression creates interesting possibilities for further development and extension of bacteriocin applications as preservatives in various food industries.

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Chapter 12
Oxidative Changes and Their Control in Meat and Meat Products

Karl-Otto Honikel

Introduction

Oxygen is a rather reactive molecule and is able to combine with many compounds within a living organism and food. But due to this reactivity it is also essential for animal life because reactions with oxygen provide the tissues with chemical energy. But the main constituents of muscular and fatty tissues are in a healthy live animal rather unsusceptible for unwanted oxidative changes. The reason is the presence of antioxidative substances in sufficient concentrations which despite the prevailing high oxygen concentrations in the tissue control the oxidation processes.

Antioxidants are reduced chemical compounds which react with oxygen or other already oxidized constituents of tissues. In these reactions the antioxidants are oxidized. They can be either reduced again by other reduced substances or, if not possible, will loose their antioxidative character. The antioxidative compounds are either directly received via feed/food or formed in metabolism with the help of the reduced matter of the feed/food. If oxidative changes occur irreversibly in a live animal then the oxidized compounds are either degraded within the cells or removed via the bloodstream.

After death the antioxidants present in muscles like NADH (nicotine–adenine–dinucleotide), vitamins C and E and antioxidative enzymes (oxidoreductases) like catalase or glutathione peroxidase are preventing uncontrolled oxidative changes for some time postmortem as long as the necessary substrates are not oxidized themselves. The oxidized antioxidants remain in the meat. In a carcass or a piece of intact meat, however, the concentration of oxygen in the interior is low as the oxygen present at death has been used up by metabolic processes and the myoglobin in the surface layers of the meat binds the oxygen in the surrounding air and forms oxymyoglobin. Hence in contrast to the high

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oxygen concentration in a muscle of a living animal, its concentration in meat is low and should remain low during storage of meat.

The presence of oxygen cannot be excluded during the slaughter process, the meat handling and processing. During slaughter and the immediately following chilling the carcass is surrounded by air. Also unavoidable is air during wholesale or retail cutting of carcasses. Only during storage for ageing or after retail cutting, a vacuum or a modified atmosphere containing either only nitrogen or a mixture of nitrogen and carbon dioxide can be applied.

Often, however, meat in retail cuts is packaged under high oxygen concentrations as the bright red color of the formed oxymyoglobin on the surface is preferred on display of meat. Additionally, the display under light accelerates oxidative processes as oxygen is activated into its singlet state by light (see below). Due to this oxidation the antioxidative substances in the meat are used up and its antioxidative power is reduced.

During processing, on mincing, or even comminuting of meat in a bowl chopper the surface of the meat is greatly enhanced and oxygen is present throughout the mince or batter. Furthermore during the comminution process the cellular membranes are disrupted and oxygen penetrates more easily to the oxidizable compounds. This disintegration of membranes also occurs on heating of intact meat cuts. The action of oxygen during heating processes, however, can be retarded if a formation of mostly brown-looking Maillard reaction products occurs which have an antioxidative power.

A possibility for the exclusion of oxygen during the processing steps is to work under vacuum, the use of nitrogen or carbon dioxide gas as protecting gases which can be done with industrial manufacturing equipments. The addition of antioxidants is another possibility which can be applied for protection from oxidation. Especially vitamin E in feed protects the meat and meat products during storage and processing. Among others, vitamins C and E, isoascorbates, nitrites/nitrates, chelating agents, synthetic antioxidants, and natural antioxidants like spices/herbs or their extracts are used in meat product manufacturing.

Oxidative changes are not per se negative events. Oxidative changes may contribute to sensory flavor characteristics of meat and meat products. Unsaturated fatty acids can be oxidized creating a number of desirable products like ketones, aldehydes, alcohols, and acids which in small concentrations add to the flavor of the product (Farmer, 1992). This happens already during chilled storage of raw meat but mainly on heating. On storing the cooked meat in a chilled or frozen state, however, the oxidative processes continue, the concentrations increase, further reactions occur and on reheating an unpleasant flavor and even odor is noticed which is called warmed-over flavor (WOF, see Pearson, Gray, Wolzok, & Horenstein, 1983; Gray & Pearson, 1987).

Oxidation may also affect the color of meat forming the brown metmyoglobin where the Fe ion in the porphyrin ring of hem is oxidized to Fe^{3+} from Fe^{2+} which is present in the bright red oxymyoglobin and the red myoglobin without oxygen. Finally, the oxidation of meat compounds to other reactive

Table 12.1 Factors influencing the oxidation in meat and meat products

Status	Process steps
Animals	Feed stuffs
	Health status
	Slaughter process
Meat	Storage time
	Storage temperature
	Size of cut
	Packaging of cuts (MAP, vacuum)
	Display under light
Processing	Heating of meat
	Cold storage of heated meat (WOF)
	Mincing, comminution
	Addition of antioxidants/prooxidants
	Packaging of products (MAP, vacuum)
	Display under light
	Storage time
	Storage temperature

MAP, modified atmosphere packaging; WOF, warmed-over flavour.

compounds in higher concentrations like peroxides, ketones, aldehydes, and other reactive products are regarded as cancer inducing or promoting substances (WCRF, 2007).

Thus, the use of antioxidative substances and/or oxygen exclusion measures in the processing of meat is common and necessary. They are applied for reasons of safety (shelf life), flavor, odor and color of meat, and meat products beginning with the feed, during slaughter, storage, preparation, and processing (Table 12.1).

In the live animal, the presence of antioxidative substances like vitamins C and E and compounds of herbs and spices in feed will enhance the antioxidative status. Also a healthy animal is keeping the antioxidative conditions high. In the postmortem meat, temperature, time, size of cuts and packaging, and display are factors which influence the presence and reactivity of oxygen around or in the meat. During processing by any means, from heating of meat cuts to manufacturing of meat products, the oxygen may deploy its action or may be retarded or inhibited by the various procedures during processing and storage.

Chemical Reactions of Oxygen

Induction by Light

Oxygen exists under normal conditions in the triplet electronic state ($^3O_2$). This is the low-energy state in which the two electrons with the highest energy have

$$\text{sensitizer} + \text{light} \rightarrow \text{sensitizer}_{\text{excited}} \quad \text{(reaction 1)}$$

$$\text{sensitizer}_{\text{excited}} + {}^{3}O_2 \rightarrow \text{sensitizer} + {}^{1}O_2 \quad \text{(reaction 2)}$$

$$R\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}R + {}^{1}O_2 \rightarrow R\text{-}\overset{\overset{\displaystyle OOH}{|}}{CH}{=}CH\text{-}CH\text{-}CH_2\text{-}R \quad \text{(reaction 3)}$$

Fig. 12.1 Sensitizing of oxygen

parallel spins. There exists the principle of conservation of spin angular moments in reactions. Unsaturated bonds in fatty acids and the peroxides formed thereof are both in the singlet electronic state with nonparallel electron spins. Hence the reaction of the low-energy triplet oxygen with an unsaturated bond in a lipid is rather unlikely. The triplet electronic state can, however, be changed, e.g., by light in the presence of a sensitizer-like riboflavin or others in a muscle (reactions 1 and 2 in Fig. 12.1). Singlet oxygen ($^{1}O_2$) reacts much faster (about 1500-fold) with double bonds (Gordon, 2001).

Not only oxygen can be activated by UV or visible light but also the chemical double bonds like those in unsaturated fatty acids may form radicals (R*) or radical ions with hydrogen release or electron transfer which then react with oxygen to form peroxides (ROOH, Fig. 12.2). The autoxidation starts mainly by the transfer of a hydrogen atom of the unsaturated bond to an existing radical. The unsaturated compound itself becomes now a radical (R*). The addition of oxygen to an existing radical is resulting in a hydroperoxide radical (Fig. 12.2).

A second initiation step for autoxidation is the splitting of an existing peroxide which is usually present in small concentrations in fatty tissues (Fig. 12.2). Two radicals are formed which lead to the prolongation step multiplying and creating in this way an avalanche of radicals and oxidized products if the sequence of events is not inhibited or stopped by the reaction of two radicals. As shown in Fig. 12.2, the radical from molecule R* can be transferred via a peroxide radical to another molecule R_1*.

Induction by Metal Ions

Meat, like all other living matter, contains ions of transition metals like iron, copper, cobalt, manganese, chromium, etc. They occur in widely varying concentrations of 0.5 mM for iron to, e.g., <1 μM for chromium. All of these ions exist in several states of oxidation in which they can change more or less easily. Their red/ox potential affects the velocity of autoxidation in food and the

initiation	$X^* + RH$	→	$R^* + HX$
2nd initiation	$ROOH$	→	$RO^* + OH^*$
	$2\ ROOH$	→	$ROO^* + RO^* + H_2O$
propagation	$R^* + O_2$	→	ROO^*
	$ROO^* + R_1H$	→	$ROOH + R_1^*$
termination	$ROO^* + R_1OO^*$	→	$ROOR_1 + O_2$
	$ROO^* + R_1^*$	→	$ROOR_1$
	$R^* + R_1^*$	→	RR_1

X^* is the first radical formed by light, heat, sensitizer

RH, R and R_1 are synonymous for unsaturated fatty acids

Fig. 12.2 Autoxidation sequence of reactions

further breakdown of the formed peroxides to volatile compounds important for flavor and odor. The first step of radical formation catalyzed by metal ions is induced by light, energy (heat), or enzymes (lipoxygenases). The metal ions in the lower state of oxidation (M^n) react very quickly with hydroperoxides (ROOH) as an electron donor as shown in Fig. 12.3. The metal ion turns into M^{n+1} and a radical RO* is formed. The following reaction with a second peroxide forms a second radical (ROO*) and the metal ion is reduced to M^n. The reduced metal ion can react again as it is typical for a catalyst. In both reactions shown in Fig. 12.3 two radicals are coming into existence by one metal

$$M^n + ROOH \rightarrow RO^* + OH^- + M^{n+1}$$

$$M^{n+1} + ROOH \rightarrow ROO^* + H^+ + M^n$$

$$H^+ + OH^- \rightarrow H_2O$$

Fig. 12.3 Metal ion-induced autoxidation

ion and a water molecule from H^+ and $OH^- = H_2O$ is formed. The reactions in Fig. 12.3 show that the metal ion must be in the reduced state for starting the autoxidation. The red/ox state depends on many conditions like solvent, pH, and presence of electron donors (reduced compounds) such as ascorbate or cystein. pH 5–5.5 is optimal; a pH which exists in a piece of meat. Fe ions are usually more effective than Cu ions.

Meat has a further metal-induced oxidative power. Fe ions are bound in meat to myoglobin in its prosthetic group, the hem, which is built by the porphyrin ring with a central Fe ion. The iron can change its oxidative form from Fe^{2+} to Fe^{3+} even to Fe^{4+}. In a muscle of a live animal the Fe ion is in the Fe^{2+} status (red color). Only with the low oxygen concentration postmortem the Fe ion is oxidized to Fe^{3+} (brown color) and further. The myoglobin-bound or more correct the prosthetic group (hem)-bound Fe ion which accelerates the metal-catalyzed lipid oxidation to hydroperoxides faster in comparison to free Fe ions if the meat is heated and the protein moiety is denatured (Tichivangana & Morrissey, 1985). This leads then to the above-mentioned warmed-over flavor.

The sequences of reactions are shown in Fig. 12.4. The formed H_2O_2 can react also with a free Fe^{2+} ion forming an OH* radical which can initiate lipid oxidation as shown in Fig. 12.3. The H_2O_2 reacts also with hem(Fe^{3+}) and removes via a hem$^+$(Fe^{4+}) = O (ferryl radical) at the end of the reaction sequence a hydrogen ion (proton) from an double bond in the unsaturated fatty acid (RH). The unsaturated fatty acid forms a radical R* which can react with oxygen to form a hydroperoxide (Fig. 12.2).

$$\text{hem}(Fe^{2+}) = O_2 \longrightarrow \text{hem}(Fe^{3+}) + O_2{}^{*-}$$

(bright red) (brown)

$$2O_2{}^{*-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$\textbf{(free } Fe^{2+} + H_2O_2 \longrightarrow \textbf{free } Fe^{3+} + OH^- + OH^*\textbf{)}$$

$$\text{hem}(Fe^{3+}) + H_2O_2 \longrightarrow \text{hem}^+(Fe^{4+}) = O + H_2O$$

$$\text{hem}^+(Fe^{4+}) = O + RH \longrightarrow \text{hem}^+(Fe^{4+}) = O + R^* + H^+$$

Fig. 12.4 Hem-catalyzed lipid oxidation (adapted from Monahan, 2000)

Induction by Enzymes

The mode of action of, e.g., the enzyme lipoxygenase differs from that of the nonenzymatic initiation of lipid oxidation. The rather complicated mechanism starts with an oxidation step of the unsaturated fatty acid, releasing a methylene group-bound hydrogen ($-CH_2 - \rightarrow -C^*H - + H^*$). A conjugated diene is formed (Yanishlieva-Maslarova, 2001). Then the oxygen is taking up by the enzyme, a peroxide radical is formed and after the addition of an hydrogen and creating the hydroperoxide the compound is released from the enzyme (Grossmann, Bergmann, & Sklan, 1988). Lipoxygenase initiation of lipid peroxidation requires for the enzyme an activation by a preformed hydroperoxide. Also the fatty acid must exist in free form and not bound to glycerol in a glyceride (Kanner, Haral, & Hazan, 1986).

The discussed possibilities emphasize that the primary initiation of a radical-driven fat oxidation is most likely light or temperature (heat). All the reactions described in this chapter are just a part of the many theories and proven events which exist about fat oxidation in meat. For further details and information see Monahan (2000).

Lipid Oxidation in Meat

As said above fresh raw meat in the first days postmortem exhibits an acceptable stability against oxidative processes due to concentration of antioxidants which deteriorate with time of storage leading to discoloration (metmyoglobin), rancidity, and health hazardous oxidation products. As an example, the reduction of NADH from day 1 to day 5 in meat is shown in Fig. 12.5. Freezing

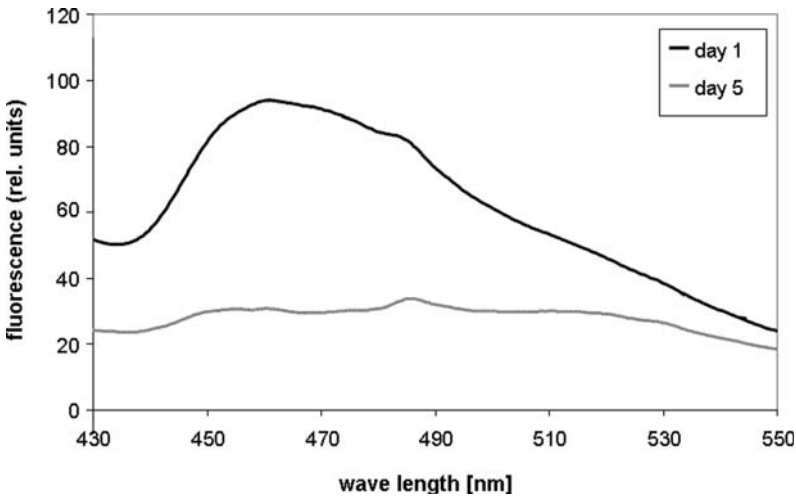

Fig. 12.5 Fluorescence of NADH in meat at 1 and 5 days postmortem (excitation wavelength 330 nm [adapted from Schneider et al., 2008])

and thawing, addition of salt, display in light and mincing are causing increased oxidative stress.

Rancidity

The most known oxidation of lipids is the sensory impression of rancidity and warmed-over flavor (WOF). Factors influencing the development of rancidity and WOF in meat and meat products, including restructured meats, have been extensively reviewed (Gray & Pearson, 1987). These include

(i) the composition and freshness of raw meat components;
(ii) cooking and/or heating of the product;
(iii) processing techniques which result in tissue membrane disintegration and subsequent exposure of the constituents to air;
(iv) storage; and
(v) various additives which may have prooxidant or antioxidant properties.

Pearson and Tauber (1984) have indicated that the freshness and bacterial quality of raw meat components play a major role in preventing and/or retarding oxidation in meat and meat products.

It is widely recognized that sodium chloride (salt) may initiate color and flavor changes in meat, but its action is still not fully understood. Early work suggested that salt catalyzed oxidation by lipoxidase or by myoglobin (Tappel, 1952). Chang and Watts (1950) reported, however, that salt had no greater effect on rancidity in the presence of hemoglobin or muscle extract than in their absence. They also demonstrated that the catalytic effect of salt depended on its concentration and the amount of moisture in the system. Aqueous salt solutions were antioxidative at concentrations of sodium chloride above 15%, but dry salt readily promoted oxidation in lard. The mechanisms of salt-induced rancidity in pork were examined by Ellis, Currie, Thornton, Bollinger, and Gaddis (1968) who reported that increasing levels of salt accelerated autoxidation. They postulated that salt may activate a component in the myofibrillar tissue which results in a change in the oxidation characteristics of adipose tissue.

The prooxidative activity of salt in processed meats can be minimized by the application of various ingredients. As a pronounced example, the antioxidative nature of nitrite in cured meats is well documented (Gray & Pearson, 1984). Also phosphates have been shown to moderate the oxidative effects of salt in pork patties (Keeton, 1983) and restructured pork (Schwartz & Mandigo, 1976).

Warmed-Over Flavor

Cooking of meat causes further oxidative stress since by heating besides membrane disintegration also antioxidative enzymes in the muscle, like catalase and

superoxide dismutase, may denature and loose their activity, while iron-containing proteins at the same time become a source of catalytic iron or, like myoglobin and hemoglobin, may be transformed into partly denaturated forms with "pseudo peroxidase" activity. Oxidative changes in heat-treated processed meat are influenced by a higher number of factors than in fresh meat, and minimization of lipid oxidation in precooked or heat-processed meat requires many factors to be considered at the same time. Such a multifactorial approach was described, defining the critical control points to be considered for processed meat (Skibsted, Mikkelsen, & Bertelsen, 1998).

The oxidative changes in cooked pieces of meat and mince occurring during chilled storage are called warmed-over flavor (WOF) as defined by Tims and Watts (1958): "WOF is the rapid development of off-flavors is in contrast to the more slowly developing rancidity encountered in raw meats or fatty tissues during refrigerated and frozen storage. Although WOF can occur in fresh meat, it most commonly occurs in meats that are cooked or in which the membranes are broken down by processes such as restructuring or grinding. Thus, any process that disrupts the integrity of the membranes encourages development of WOF." Consumers recognize WOF as an unpleasant flavor, calling it as "old, somewhat rancid or fishy." The highly unsaturated phospholipids, which are integral parts of the cellular membranes, have been identified as oxidation substrate and responsible for the development of WOF. Hence lean meats are equally exposed to development of WOF as more fatty meats (Mielche & Bertelsen, 1994).

Preprepared foods processed by heating experience a steadily increasing demand. Thus they require new production and packaging concepts. For such meals, the sensory quality of the meat is often central for the overall impression and control of the WOF is accordingly critical (Mielche & Bertelsen, 1994). Vitamin E (α-tocopherol) incorporated in the cellular structures during animal growth is found to be superior to this antioxidant-added postmortem during processing of the meat in preventing lipid oxidation processes (Mitsumoto, Arnold, Schaefer, & Cassens, 1993) and also in limiting the formation of cholesterol oxidation products in processed meats as discussed later (Monahan et al., 1992). Cholesterol oxidation products in the diet may constitute a health risk in promoting atherosclerosis and other lifestyle-related diseases (Maerker, 1980).

Cured meat products are surprisingly stable against lipid oxidation and development of WOF as long as the cured meat pigment is not oxidized by the combined action of oxygen and light (Skibsted, 1992). Protection of cured meat against oxidation seems to be possible if the meat used for curing is selected from animals raised on feed with an increased level of vitamin E or if the presence of oxygen is minimized during storage.

The catalytic effects of iron in meat on WOF are well documented. In the 1960 s myoglobin was viewed as the major catalyst of lipid oxidation (Tappel, 1962). However, studies by Sato and Hegarty (1971), Love (1972) and Igene (1978) revealed that nonhem iron, rather than hem iron, was the active catalyst

responsible for the rapid appearance of WOF in cooked meat. By the heating process, the iron in the hem moiety of myoglobin becomes "free" due to the denaturation of the protein moiety. Free iron is known to decompose lipid hydroperoxides, forming very reactive alkoxy radicals for the propagation reactions (Ingold, 1962). Also, the mechanism proposed by Tappel (1962) depends on the presence of lipid hydroperoxides which react with hem compounds and undergo homolytic decomposition. The ability of hem pigments and nonhem iron to accelerate the propagation step of the free radical chain mechanism (see Figs. 12.3 and 12.4) can explain the rapid rate of lipid oxidation in cooked meats (Tichivangana & Morrissey, 1985). Kanner and Harel (1985) have demonstrated that metmyoglobin, when activated by hydrogen peroxide, will initiate membrane lipid oxidation. They proposed that autoxidation of oxygenated hem pigments (oxymyoglobin and oxyhemoglobin) leads to formation of met-hem proteins and the superoxide radical (O_2^-), which dismutates to form hydrogen peroxide (see Fig. 12.4). A reactive porphyrin ferryl radical ($P - Fe^{4+} = 0$) results from the reaction of metmyoglobin and methemoglobin with hydrogen peroxide.

Rhee, Ziprin, and Ordoñez (1987) studied the mechanism of lipid oxidation in meat systems and concluded that the hem pigment system (Fe III-Mb / H_2O_2, see Fig. 12.4), regardless of how it exerts its prooxidative effect, plays a major role in the catalysis of lipid oxidation in raw and cooked meat. They suggested that hydrogen peroxide-activated metmyoglobin was the primary initiator of lipid oxidation in raw meat, and that nonhem iron, released from metmyoglobin by the action of hydrogen peroxide, was the major catalyst of lipid oxidation in cooked meat.

Cholesterol Oxides

A very thorough study of the oxidation of lipids and cholesterol has been done in the thesis of Münch (2003) wherein he investigated various meat species. In Table 12.2 the oxidation at fatty acids measured as malondialdehyde (TBARS values) by heating of pork chops and roast beef is shown. Pan frying and water cooking enhanced the TBARS values by a factor of 3–4.

If the pork chops are reheated by cooking in water after 1–7 days of chilled storage the values of TBARS in pork chops of cooked meat rise immediately

Table 12.2 Concentration of malondialdehyde (TBARS value) (mg/kg muscle) in raw meat and after different treatments and cuts of different species (adapted from Münch, 2003) to well done (~80°C); the raw meat was used about 5 days post mortem

Treatment	Pork chop	Roastbeef
Raw	0.09	0.18
Pan fried	0.42	0.55
Water cooked	0.44	0.33

Table 12.3 Concentrations of malondialdehyde (TBARS values) after chilled storage lean pork chops and reheating by cooking in water bath or in microwave and stored at 8°C

Sample	TBARS (mg/kg product)
Reheating by cooking after storage for	
1 day	0.59
2 days	0.64
7 days	1.45
Reheating by microwave after storage for	
1 day	0.82
2 days	1.41
7 days	3.09

after cooking with 0.44 mg TBARS/kg meat (Table 12.2) and to 0.59/0.64/1.45 mg TBARS/kg meat after 1/2/7 days of chilled storage. If the reheating is done by microwave, then the values are even higher of 0.82/1.41/3.09 mg TBARS/kg meat after 1/2/7 days of storage (Table 12.3).

The results exhibit two things. Chilled storage of heated meat enhances the oxidation of fatty acids in muscle more than a storage of raw meat for some days as shown in Table 12.2 with raw meat at 5 days postmortem. But besides the storage time, the heating treatment is also important. Microwave heating nearly doubles the TBARS values of those of cooking in water.

Not only the fatty acids, but also the cholesterol is oxidized to a number of oxides which are shown in Table 12.4. In this table, different pork cuts have been analyzed for cholesterol oxides in raw, pan fried (80°C), and cooked in water (ca. 85°C). In the raw meat of all cuts, all oxides are lower than that in the heated samples. The minced meat has higher concentrations of 7 α-diol/7 β-diol and triol already in the raw meat which are further enhanced during the heating processes. The minced meat has a larger surface and thus the oxygen has an easier access to the phospholipids. The two different heating regimes (pan fried or cooked in water) enhanced in general the concentrations of the oxides; the cooking in water (no Maillard reaction products possible) exhibits higher oxide concentrations in most cases. If the pan-fried sample are stored up to 7 days and reheated, either by frying or by microwave heating (Table 12.5), then the observed results show a very similar behavior to those of Tables 12.2 and 12.3. Heating enhances cholesterol oxides and TBARS values, both during chilled storage, and reheating by frying, cooking, or microwave heating.

Figure 12.6 shows the results in a graph. It is apparent that some cholesterol oxides are increasing by factors of 50–60 in 7 days, others by factors of about 10; some of the possible oxides are not changed very much and remain altogether low in concentration.

The changes of cholesterol oxides during frozen storage at −20°C are shown in Table 12.6. Storage for 30 weeks leads only to slight increases of most cholesterol oxides. The concentrations at 30 weeks are much smaller than after chilled storage for 7 days without reheating. Reheating let the cholesterol

Table 12.4 Concentration of cholesteroloxides (μg/kg muscle) in raw and heated pork cuts (adapted from Münch, 2003); raw cuts were analysed at 5 days post mortem, heated samples at the day of experiment

Cholesterol oxides		Eye of hind leg	Leg bottom	Rump	Mince	Shoulder blade	Chop
7α-diol	Raw	26	23	33	102	30	41
7β-diol		24	27	35	111	35	43
β-epoxide		n.d.	n.d.	n.d.	n.d.	n.d.	38
α-epoxide		5	35	6	n.d.	26	32
Triol		16	10	42	83	18	41
25-diol		11	18	22	23	19	17
7-keto		12	52	10	12	110	88
7α-diol	Pan fried	41	36	33	147	24	42
7β-diol		48	47	40	192	28	44
β-epoxide		n.d.	n.d.	n.d.	n.d.	n.d.	32
α-epoxide		40	77	30	n.d.	27	32
Triol		14	24	29	80	16	34
25-diol		20	16	27	34	20	21
7-keto		58	67	22	45	124	98
7α-diol	Cooked in water	97	21	93	139	33	46
7β-diol		122	32	134	228	44	56
β-epoxide		n.d.	n.d.	n.d.	n.d.	34	56
α-epoxide		44	34	71	n.d.	88	32
Triol		8	10	37	53	20	34
25-diol		25	18	30	37	21	24
7-keto		106	56	74	22	205	104

n.d., not detectable.

Table 12.5 Concentrations of cholesterol oxides in lean pork chops, reheated by pan frying or microwave after storage for 1, 2 and 7 days at 8°C (adapted from Münch, 2003)

	Cholesteroloxides (μg/kg muscle)							
	7α-diol	7β-diol	β-epoxide	α-epoxide	20α-diol	triol	25-diol	7-keto
Reheating by frying after storage for								
1 day	156	271	134	71	n.d.	41	35	353
2 days	459	760	351	133	n.d.	65	52	1004
7 days	974	1476	393	300	n.d.	59	59	1424
Reheating by microwave after storage for								
1 day	187	314	116	78	n.d.	59	54	463
2 days	441	721	220	124	n.d.	60	51	990
7 days	1009	1476	354	246	4	87	63	1506

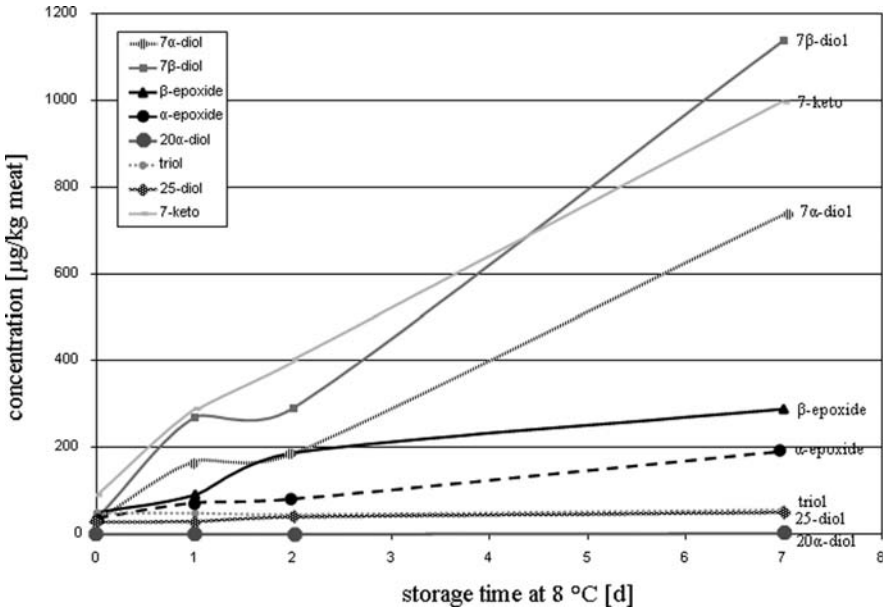

Fig. 12.6 Changes of cholesterol oxides during chilled storage of pan-fried pork chops (for details see Table 12.5)

concentrations rise to those mentioned in Table 12.5. Due to the toxicological behavior of some cholesterol oxides, a reheating of chilled meat should be avoided if possible, if there is no protection against oxidation provided by other means as discussed below.

Prevention of Lipid Oxidation in Meat

Vitamin E Supplementation

If vitamin E is added to animal feed, it becomes an integral part of cellular membranes, in contrast to the added vitamin E to a product during processing (Bertelsen, Jensen, & Skibsted, 2000). The location of the α-tocopherol, the main vitamin E compound, is in close proximity to the phospholipids as primary oxidation substrate and to the membrane cholesterol. It is the assumed basis for the pronounced antioxidative effects of vitamin E which is generally achieved.

In the review by Gray, Goman, and Buckley (1996), it is surprisingly concluded that feeding supranutritional levels of vitamin E to cattle does not provide any distinctive benefits for precooked beef. As said above, heating of meat liberates catalytically active iron from the hem group of myoglobin and

Table 12.6 Changes of cholesterol oxide concentrations (μg/kg meat) during frozen storage (−20°C) in raw, pan fried and cooked pork chops (vacuum packaged), adapted from Münch (2003)

Treatment	Raw				Pan fried					Cooked in water				
Storage weeks	0	4	11	30	0	4	11	20	30	0	4	11	20	30
7α-diol	47	49	69	75	52	64	80	86	86	57	76	73	92	187
7β-diol	50	58	73	71	49	83	99	94	94	74	100	93	135	213
β-epoxide	31	28	n.n.	33	13	13	13	70	8	60	69	32	86	70
α-epoxide	27	23	11	47	26	39	42	90	35	25	49	58	57	77
Triol	32	26	25	37	18	21	26	49	39	20	24	30	33	75
25-diol	17	18	19	20	14	18	20	14	21	15	23	22	23	23
7-keto	91	95	93	120	106	110	148	146	154	118	132	136	195	343

from other iron-containing proteins and transforms myoglobin and hemoglobin into prooxidative species, resulting in accelerated oxidation of lipids in the membranes (Geileskey, King, Coste, Pinzo, & Ledward, 1998; Mielche & Bertelsen, 1994). The higher content of hem and hem iron in beef and pork (Schricker & Miller, 1983) results in a higher load of prooxidative species in precooked products from these species compared to, for example, poultry and provides part of the explanation for the relatively lower vitamin E effect in beef and pork compared to poultry. The importance of prooxidative species originating from meat myoglobin and other iron-containing proteins is also evident from the studies performed with chicken, where a lower protection by vitamin E in general has been found for dark thigh meat compared to white breast meat (Jensen, Skibsted, Jakobsen, & Bertelsen, 1995; Galvin, Morrisey, & Buckley, 1998), despite the fact that the accumulation of vitamin E is higher in dark meat compared to white meat.

Protection in Meat

Supplementation of feed with vitamin E can be done in different ways. It can be done continuously in smaller amounts over the whole fattening period or within the last few weeks in higher doses. Table 12.7 shows such an experiment with pigs. Pigs were fed from about 27 kg live weight until slaughter (ca. 110 kg live weight) for about 100–110 days with no additional supplementation of vitamin (natural content of feed was 32 mg vitamin E/kg feed) and 100 or 200 mg vitamin E/kg feed, equivalent to about 22–44 g added vitamin E in total over the whole feeding period. A fourth group received additionally in the last 3 weeks before slaughter 1.2 g vitamin E/day equal to 21×1.2 g = 25.2 g total which is in between the feeding of groups 2 and 3 (Rosenbauer & Honikel, 2002, personal communication).

Figures 12.7 and 12.8 show that the concentrations of vitamin E increase in loin muscle and back fat in all supplementation regimes and are about twofold enhanced by 100 mg vitamin/kg feed respective 1.2 g/day in loin (2.5 vs. 5 μg vitamin E/kg and 10 vs. 20 μg vitamin E/kg back fat).

It is evident that the fatty tissue contains about five times higher vitamin E concentrations than the lean loin muscle (ca. 2% fat). As shown in Figs. 12.7 and 12.8 the additions of 200 mg vitamin E/kg to the feed enhanced the vitamin

Table 12.7 Supplementation of feedstuff with vitamin E

Group	Control	II	III	IV
Number of samples	13	13	12	12
Vitamin E-addition[a] (mg/kg feed dry matter)	0	100	200	0
Vitamin E-addition[a] in the last 3 weeks (g/day) prior to slaughter	0	0	0	1.2

[a] Additional to the basic content of 32 mg vitamin E/kg feed (covering the requirement).

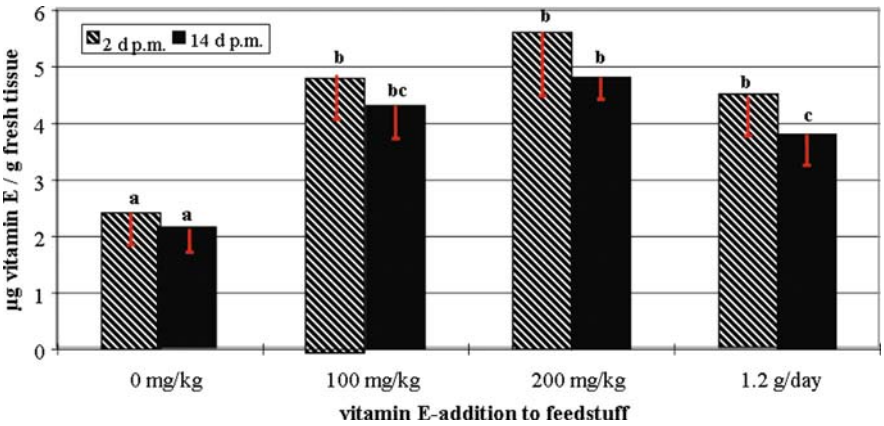

Fig. 12.7 Vitamin E level in fresh (2 days postmortem) and for 14 days at 6°C stored pork loin muscle (the *line* in the column indicates the lower half of the standard deviation, N = 10)

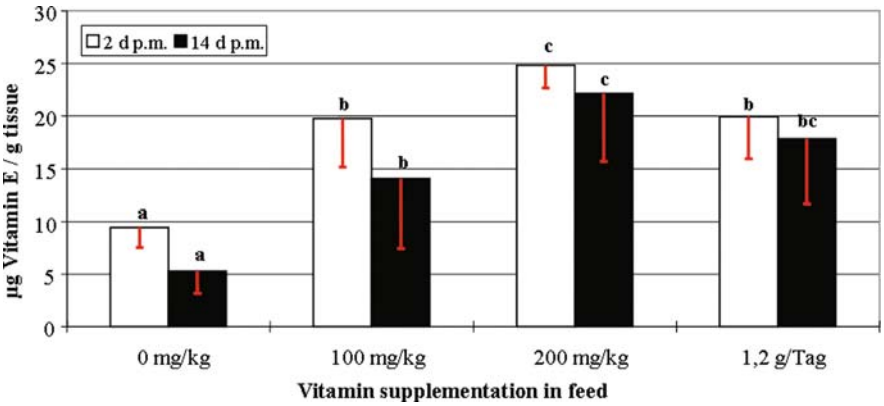

Fig. 12.8 Vitamin E concentration in fresh (2 days postmortem) and for 14 days at 6°C stored pork back fat (the *line* in the column indicates the lower half of the standard deviation, N = 10)

E concentration in loin and back fat by further 15–25%. During chilled storage for 14 days the vitamin E concentration fell by 10–25% (Rosenbauer & Honikel, 2002, personal communication). Figure 12.9 shows another experiment where 1 g vitamin E was fed per day for 1, 2, and 3 weeks prior to slaughter. Without supplementation the concentration in the muscle and liver was rather low. In liver and back fat it increased strongly with the time of supplementation.

The higher values of vitamin E in the tissues reduced the oxidation products measured as TBARS values from about 0.10 mg malondialdehyde/kg loin tissue with 2 µg vitamin E/g tissue to 0.06 mg malondialdehyde with 7 µg vitamin E/g loin tissue. With the time of storage, the TBARS increase and the values are the highest when the vitamin E concentration of tissue is the lowest (Fig. 12.10).

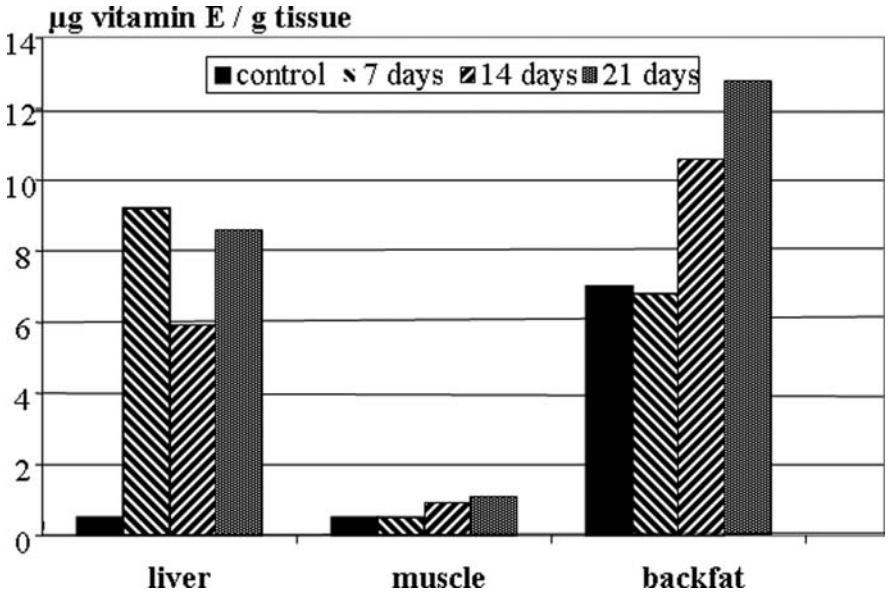

Fig. 12.9 Supplementation of pig feed with 1 g vitamin E per day for 1–3 weeks prior to slaughter and its influence on the concentrations in liver, loin muscle, and back fat of pig (N = 9 animals)

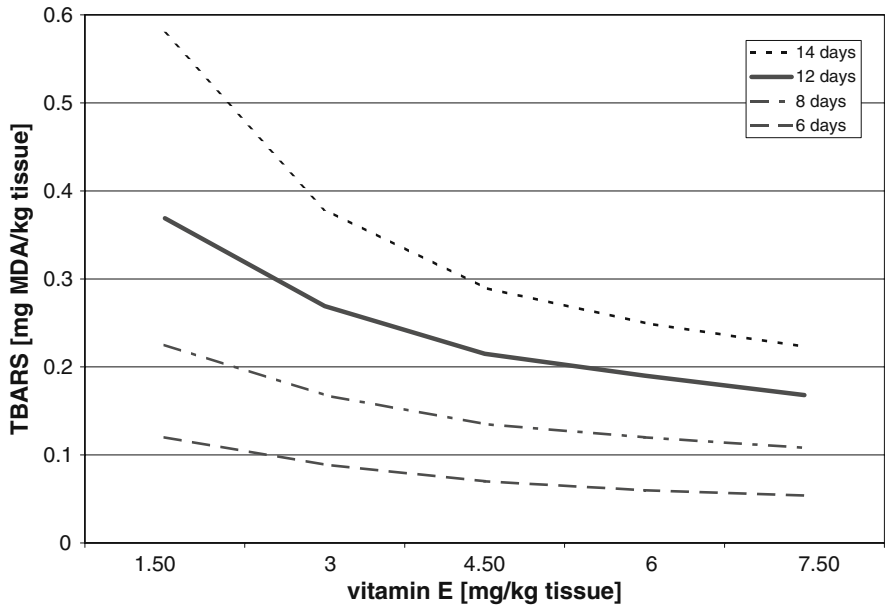

Fig. 12.10 Vitamin E level and TBARS values in pork loin muscles at storage at 4°C for 14 days (N = 10)

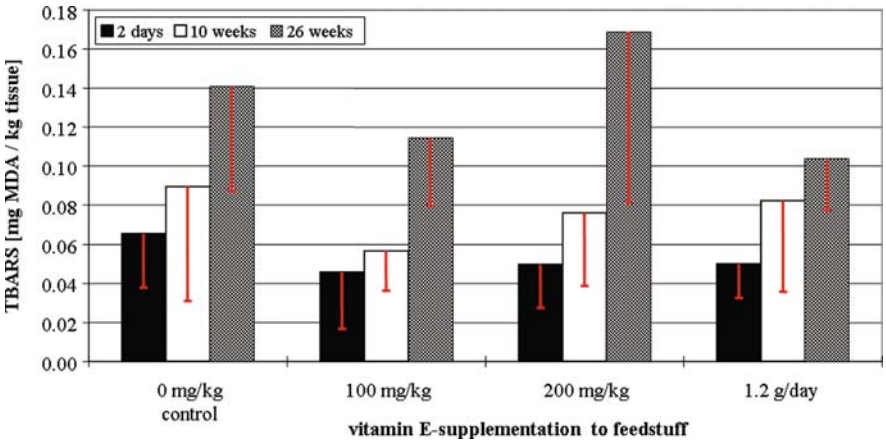

Fig. 12.11 Course of TBARS values development in pork back fat during frozen storage for 26 weeks (at −20°C, vacuum packaged; N = 10; the line in the column indicates the lower half of the standard deviation)

The results of Fig. 12.10 show that during a normal shelf life of fresh pork (<6 days) does not lead irrespective of vitamin E concentration to a sensory appearance of rancidity as the TBARS values are <0.15 mg malondialdehyde/kg fresh unsalted tissue.

In frozen back fat, the TBARS values also increase despite higher vitamin E concentrations in the tissue (Fig. 12.11). Whereas after 10 weeks the increase is rather small (0.06–0.08 mg malondialdehyde/kg tissue in control samples) the further storage for additional 16 weeks resulted in a strong increase of TBARS values. The feed supplementation of 100 respective 200 mg vitamin E/kg has no protective effect any more at 26 weeks of frozen storage.

Protection in Meat Products

The production of raw ham and salami-type sausages takes months. Figure 12.12 shows that the concentration of vitamin E is high in raw hams even after 6 months; the vitamin E supplementation effect is still clearly visible.

The TBARS values (Fig. 12.13) are in the control samples at the edge of sensory detection at 6 months of ripening. Supplementation leads to lower values at 3 months. At 6 and 9 months there existed no effect of supplementation any longer. But the TBARS values are in all cases below sensory detectable values (<0.3 mg malondialdehyde/kg product) in salted products where the limit of sensory detection is higher than in unsalted fresh meat. Interestingly, in salami-type sausages the vitamin E concentration increased during storage up to 29 weeks due to the considerable weight loss of >30% (Fig. 12.14). Despite the considerable vitamin E concentration at 29 weeks, the sausages were

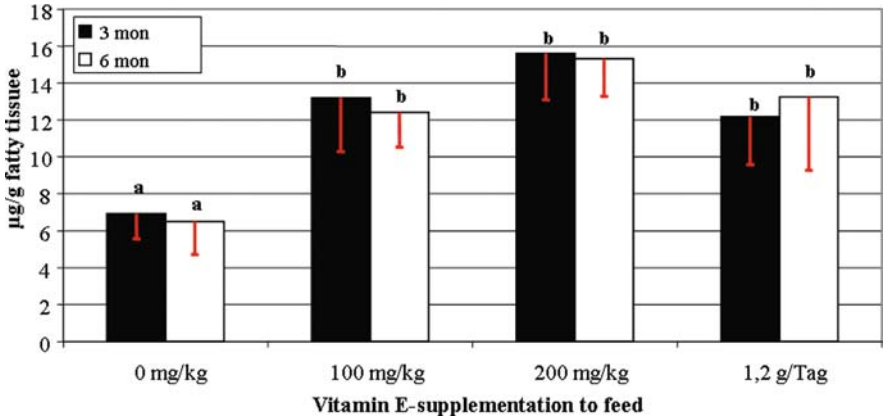

Fig. 12.12 Vitamin E concentrations in the fat of raw hams after 3 months fermentation and further 3 months (at 6 months in total) of storage (the *line* in the column indicates the lower half of the standard deviation, N = 6)

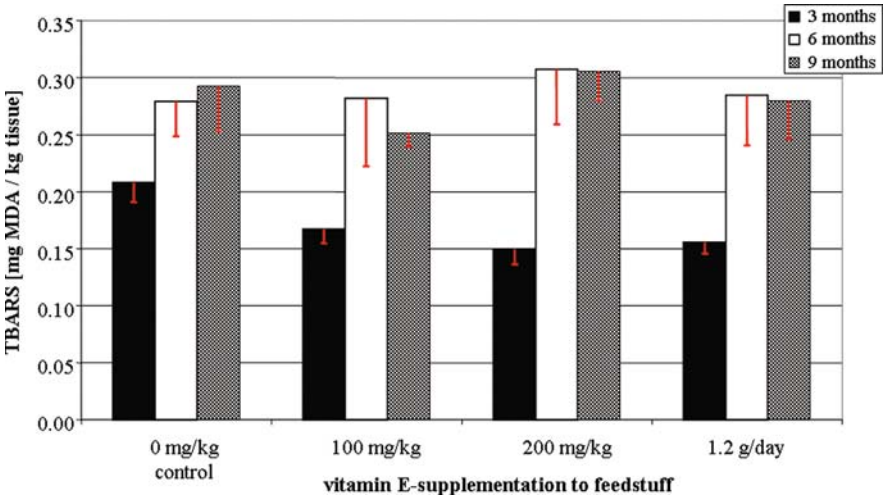

Fig. 12.13 TBARS values in the lean tissue of raw hams after 3 months of fermentation and further 6 months (in total 9 months) of storage at 12°C in vacuum ($N = 6$, the *line* in the column indicates the lower half of the standard deviation)

evaluated sensorial as rancid despite low TBARS values (Honikel & Rosenbauer, 1998). In consequence, it means that vitamin E protects against rancidity but not under all especially long-time storage conditions.

The short shelf life of cooked ham causes no problems in this respect. The supplementation led to higher and stable vitamin E concentrations (Fig. 12.15). The constant concentration at 3–4 and 13 days is most likely due to the

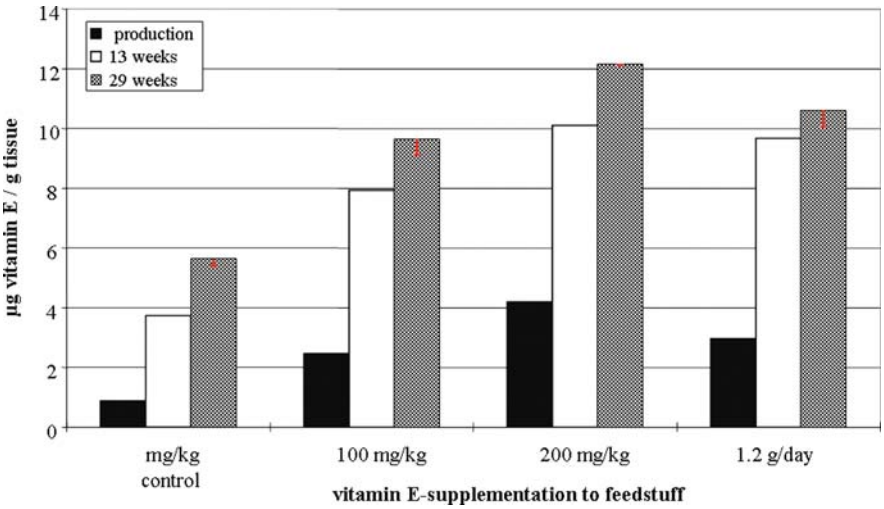

Fig. 12.14 Vitamin E concentrations in salami-type sausages immediately after start of production and after 13 and 29 weeks of ripening and storage at 12°C in vacuum package ($N = 2$; the *line* in the column indicates the lower half of the variation)

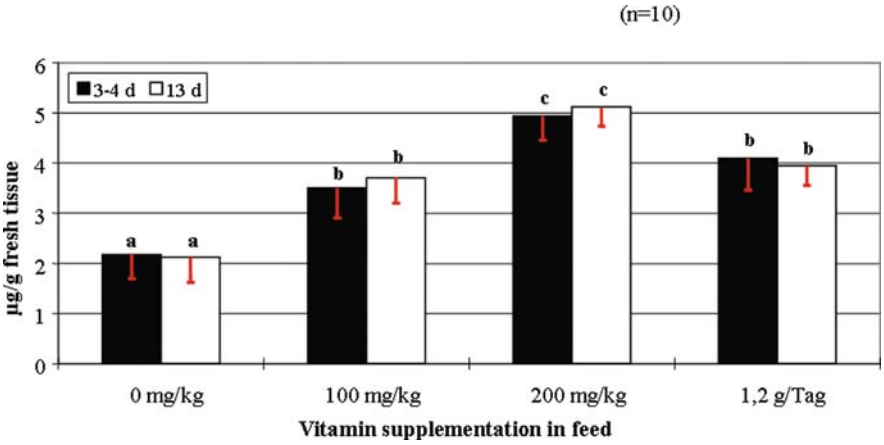

Fig. 12.15 Vitamin E concentration in cooked ham after manufacturing (3–4 days) and storage for altogether 14 days at 5°C ($N = 10$; the *line* in the column indicates the lower half of the standard deviation)

antioxidative action of the nitrite present in the product which was about 100 mg nitrite/kg addition and about 20–30 mg nitrite/kg left after heating. There was no rancidity development neither in the control samples nor in the supplemented ones detected (Rosenbauer, 2002).

In conclusion: In raw meat, cooked ham and raw hams (the latter two contained nitrite) enhanced vitamin E concentrations are not needed for

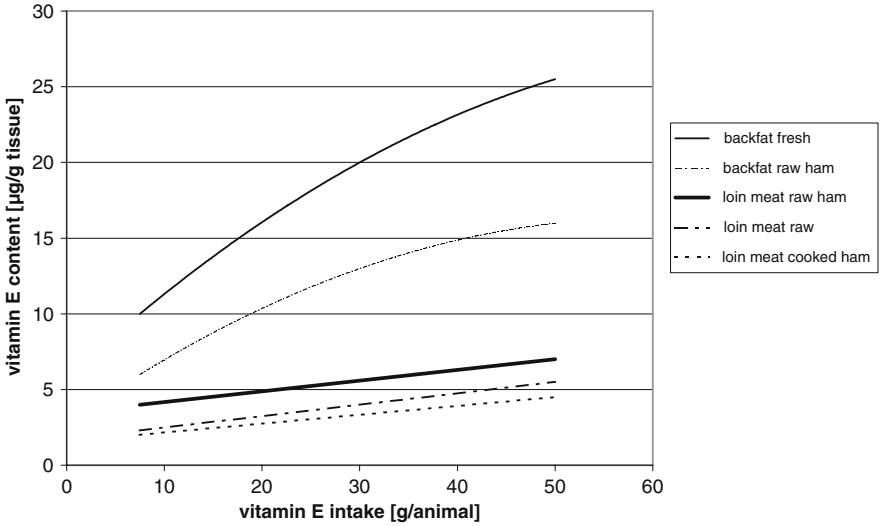

Fig. 12.16 Vitamin E intake of pigs by feed and tissue concentrations of fresh pig meat and back fat and in processed products

sensory protection against rancidity. Salami-type sausages, if stored for long periods, are not protected against rancidity. WOF, however, can be prevented by vitamin E.

The vitamin E concentration from a nutritional point of view is enhanced in fresh meat and fat (Fig. 12.16). The addition of 1.2 g/day for 3 weeks or 100 mg respective 200 mg/kg feed with 20–45 g total addition during the feeding period led to an increase from 10 to 20/25 µg vitamin E/g fatty tissue and from 2 to 6 µg vitamin E/g muscular tissue. Processing did not change the increase to a large extent.

Other Measures Against Oxidation

Many studies have indicated that WOF development in meat products can be effectively controlled or retarded by the use of antioxidants or a proper packaging. These compounds can be used singly or in combination and can range from synthetic antioxidants to compounds in natural foods like herbs and spices whose structures are not always fully elucidated. In fresh meat the use of the compounds in the following chapters are prohibited or limited.

Packaging

Harte (1987) reviewed packaging techniques and indicated that the selection of a packaging system can significantly influence the oxidative stability of meat

and meat products. Headspace control techniques (vacuum packaging, gas flushing and shrink- and skin-packaging), when used in conjunction with good oxygen and light barriers, can effectively control oxidative rancidity. Kingston, Monahan, Buckley, and Lynch (1998) reviewed the effectiveness of several packaging systems in controlling oxidation in precooked meat products. Packaging meat products in good oxygen-barrier materials can significantly retard autoxidation. The most protecting packaging materials are air tight metal and glass containers, the latter must be stored in the dark as light may induce radicals which also may lead to sensory changes. Antioxidant-impregnated films have also been used at the research level with some success in minimizing lipid oxidation in selected meat items.

Maillard Reaction Products

The antioxidant activity of Maillard reaction products is well established. For a review see Pokorny and Schmidt (2001). Rhee (1987) interestingly reported that the use of extracts from over-cooked, retorted, or pressure-cooked meat which contained brown Maillard products may not be economically feasible unless meat animal parts of little economic value are used to prepare the extracts.

Chelating Agents

Phosphates are usually added to processed meats because they increase the water-holding capacity and yield of the finished product. The addition of phosphates to cooked meats also delays or prevents lipid oxidation (Sato & Hegarty, 1971). Ortho-, Pyrophosphates, tripolyphosphates, and hexametaphosphates all offer protection but to a different extent. Phosphates appear to prevent autoxidation by chelating the heavy metal ions (Tims & Watts, 1958).

Other chelating agents have been shown to be effective as inhibitors of oxidation, presumably because of their ability to sequester transition metal ions like those of iron and copper. Liu and Watts (1970) demonstrated many years ago that ethylenediamine tetraacetic acid (EDTA) prevented Fe^{2+}-catalyzed oxidation in raw beef, while Sato and Hegarty (1971) showed that EDTA, at a concentration of 2.5 mg/g, suppressed lipid oxidation in cooked ground beef. These investigators concluded that EDTA effectively chelated free iron and thereby significantly reduced lipid oxidation in cooked meat. Although EDTA (E-385) has provided a good tool for studying the role of heavy metal ions in lipid oxidation, it has not been approved for commercial use in meat products.

Citric acid and citrates have also been evaluated as antioxidants in meat systems. Sato and Hegarty (1971) reported minimal inhibition of lipid oxidation in cooked ground beef when sodium citrate was added to the level of 5 mg/g. Macdonald, Gray, and Gibbins (1980) demonstrated that citric acid reduced

TBA numbers in refrigerated hams when used at the 1000 mg/kg level. However, this compound was not as effective as 50 mg/kg of nitrite.

At low levels (<100 mg/kg) ascorbic acid and ascorbate have been shown to catalyze WOF development in meat products (Sato & Hegarty, 1971). However, at levels in excess of 1000 mg/kg, ascorbate or isoascorbate is an effective inhibitor of oxidation. Sato, Hegarty, and Herring (1973) suggested that high levels of ascorbic acid shifted the balance between ferrous and ferric iron and acted as an oxygen scavenger. Kanner et al. (1986) demonstrated that iron in the presence of ascorbic acid stimulates membrane lipid peroxidation in muscles, presumably through the involvement of hydroxyl radicals. A synergistic relationship between ascorbic acid and phosphates in inhibiting lipid oxidation in meats was demonstrated a long time ago by Tims and Watts (1958) and Sato and Hegarty (1971). The latter investigators theorized that ascorbic acid functions by keeping a part of the iron in the reduced state. The combined actions of phosphates, ascorbate (or isoascorbate), and nitrite assist in explaining the virtual absence of WOF in cured meats.

Nitrite/Nitrate

Nitrite is limited to the use in meat products. Sato and Hegarty (1971) reported that nitrite completely eliminates WOF at a rather high level of 2000 mg/kg and delays the development of WOF at the low level of 50 mg/kg. Fooladi, Pearson, Coleman, and Merkel (1979) demonstrated that a nitrite concentration of 150 mg/kg added to meat inhibited WOF development in cooked meat, with a twofold reduction of TBARS numbers for beef and chicken, and a fivefold reduction for pork. Nitrite is easily oxidized to nitrate and acts this way as an oxygen scavenger (Honikel, 2008). Some other possible mechanisms include

(i) The formation of a stable complex between the hem pigments and the nitrite, thereby preventing the release of nonhem iron and its subsequent catalysis of lipid oxidation (Igene, Yamauchi, Pearson, & Gray, 1985; Morrissey & Tichivangana, 1985);
(ii) The formation of inactive "chelates" between nitrite and metal ions such as ferrous ions, thus rendering them unavailable for catalysis of oxidation reactions (Igene et al., 1985; Morrissey & Tichivangana, 1985); and
(iii) The formation of nitric oxide myoglobin which has antioxidant properties per se (Kanner, Ben-Gara, & Berman, 1980; Morrissey & Tichivangana, 1985). Regardless of the mechanism of nitrite in preventing oxidation and WOF development in meat, there is little doubt about its effectiveness in decreasing lipid oxidation (Sato & Hegarty, 1971; Gray & Pearson, 1984, 1987).

Smoking

The antioxidant activity of smoke is provided by a number of compounds, including phenols, phenol aldehydes, and organic acids (Toth, 1984). Phenols with high boiling points, such as 2,6-dimethyoxyphenol and 2,6-dimethyoxy-4-ethylphenol, are particularly effective (Pearson & Tauber, 1984).

Synthetic Antioxidants

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and several other synthetic phenolic antioxidants have been widely studied in meat systems and, in general, have been shown to be effective in retarding lipid oxidation. Greene (1969) reported that BHA and propyl gallate (PG) offered substantial protection against oxidation of fresh meat pigments and effectively inhibited lipid oxidation in raw ground beef. Greene, Hsin, and Zipser (1971) further demonstrated that BHA or PG prevented lipid oxidation and reduced pigment oxidation in ground beef for up to 8 days of refrigerator storage. When a combination of antioxidants and ascorbic acid was used, both lipid and pigment oxidation were effectively retarded.

Natural Antioxidants

Many studies on lipid oxidation in meats have focused on the antioxidant activity of naturally occurring substances. These substances include various edible products from spices and herbs. Houlihan and Ho (1985) and Rhee (1987) have reviewed the antioxidative nature of these substances in some detail.

Many spices and herbs have been shown to function as antioxidants in fats and oils and in model food systems (Yanishlieva-Maslarowa & Heinonen, 2001). Rosemary, for example, contains a number of compounds possessing antioxidant activity, including carnosol, rosmanol, rosmariquinone, and rosmaridiphenol. Already in 1985, Barbut, Josephson, and Maurer demonstrated that a rosemary oleoresin, when added to turkey breakfast sausage at the 20 mg/kg level, produced an antioxidative effect and did not adversely affect overall palatability of the product. The authors concluded that incorporation of rosemary oleoresin in meat products can substantially suppress lipid oxidation and increase shelf life at refrigerated temperatures.

Concluding Remarks

The chemically reactive oxygen, necessary for the energy turnover in living organisms, can show detrimental effects in meat and meat products during processing and storage. Rancidity, WOF development, cholesterol oxidation,

and the loss of bright red color of fresh meat are the unwanted changes. These changes may appear to the consumers primarily as unpleasant sensorial deteriorations, but they can also be health hazardous. Radicals may initiate cancer; cholesterol oxides are held responsible for the development of artheriosclerosis. Hence the prevention or retardation of oxidative changes is required. The century-old methods of using herbs, spices, and nitrate/nitrite and the creation of the brown-reducing Maillard reaction products are accompanied today by oxygen exclusion in vacuum or MAP packaging, the use of natural antioxidants in feed of animals or during processing, ascorbic acid (vitamin C) vitamin E or phosphates and citrates, or the addition of synthetic antioxidants.

These measures are necessary as the shelf life of meat, meat preparations, and meat products has been extended largely in comparison to half a century ago. Usually a combination of several measures is necessary in order to safeguard a product during its shelf life. But there is always a limit of shelf life as antioxidants due to the nearly ubiquitous presence of oxygen even in small concentrations leads to their oxidation. Only air tight metal or glass containers, the latter in the dark, prevent the presence of oxygen.

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Chapter 13
Polycyclic Aromatic Hydrocarbons in Smoked Meats

Peter Šimko

Introduction

Meat smoking belongs to the oldest food technologies that has been used by mankind at minimum for 10,000 years. Probably a protection against canines led a man to hung a catch over the fire and from this time the smoking has started to be widely used not only for production of smoked products with a special organoleptic profile, but also for inactivating effects on enzymes and microorganisms. So far, techniques of smoking have been gradually improved and various procedures have been developed in different regions for treating meat and fish. Nowadays, the technology is used mainly for enrichment of foods with specific taste, odor, and appearance to be demanded widely on the market. On the other hand, the role of the preservative effects is going down gradually with regard to the latest trends in alternative preservation procedures. Today it is supposed that the technology is applied in many forms to treat 40–60% of the total amount of meat products (Sikorski, 2004) and 15% of fish (Stoyhwo & Sikorski, 2005).

Principles of Smoking

In general, smoke is a polydisperse mixture of liquid and solid components with diameters of 0.08–0.15 μm in gaseous phase of air, carbon oxide, carbon dioxide, water vapor, methane, and other gases. Smoke has a variable composition which depends on various conditions such as procedure and temperature of smoke generation, origin and composition of wood, water content in wood, etc. (Sikorski, 2004). So far, up to 1100 various chemical compounds have been identified and published in literature (Wilms, 2000). The smoking treatment

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itself is based on successive deposition of compounds such as phenol derivates, carbonyls, organic acids and their esters, lactones, pyrazines, pyrols, and furan derivates (Maga, 1987) on a food surface with their subsequent migration into a food bulk. Smoke is generated during a thermal combustion of wood, consisted roughly of 50% cellulose, 25% hemicelulose, and 25% lignin, at a limited access of oxygen. The thermal combustion of hemicelluloses, cellulose, and lignin proceeds at 180–300, 260–350, and 300–500°C, respectively. However, the decomposition of the wood components processes also at temperatures reaching up to 900°C and in large excess of oxygen even up to1200°C. The smoke produced at 650–700°C is richest in components able to impart desirable organoleptic properties of treated products. The temperature for the generation of smoke can be decreased by increasing the humidity of woods (Tóth & Potthast, 1984). The quantitative composition of smoke depends not only on the kind of wood used, on the temperature of the generation and the excess of oxygen, but also on cleaning procedures of smoke applied immediately after its generation (Sikorski, 2004).

Traditional Procedures of Smoking

After the generation, smoke is driven into a kiln, while its temperature is going down, that is accomplished by partial condensation of smoke components (especially compounds with high boiling point) in pipes, walls, or foods, respectively. The rate of smoke deposition depends on the temperature, humidity, volatility, and velocity of a smoke stream. When the smoke comes into contact with the food surface, the smoke treatment procedures are divided into three modes, related to the temperature of smoke, as follows:

1. Cold smoking – with the temperature of the smoke between 15 and 25°C (used for aromatization of uncooked sausage, raw hams, and fermented thermally not treated salami).
2. Warm smoking – with the temperature between 25 and 50°C (used for aromatization, alternatively mild pasteurization of frankfurters sausages, meat pieces, and gammon).
3. Hot smoking – with the temperature between 50 and 85°C (used for both – aromatization and thermal treatment of hams, salami, sausages, etc.).

To achieve a rich deep brown coloring on the surface and very strong aroma profile formation, the time of smoking has to be considerably prolonged. Suchlike products are frequently termed as "black-smoked" or "farmhouse-smoked", respectively. However, these products contain far more high contents of polycyclic aromatic hydrocarbons (PAH) (Wilms, 2000; Šimko, Gombita, & Karovičová, 1991). "Wild" smoking at uncontrolled technological conditions and non-existing legislative measures, what is typical especially for households and developing countries, leads to enormous PAH contents in smoked foods (Afolabi, Adesulu, & Oke, 1983; Alonge, 1987, 1988).

Alternatives to Traditional Smoking Procedures

A Kansas pharmacist Wright developed and patented a first liquid smoke flavor (LSF) to be prepared from primary smoke condensate in the late of 19th century. The usage of LSF has important advantages – considerably reduces the time which is necessary to reach required organoleptic profile of flavored foods as well as makes it possible to control more effectively "addition" of contaminants, including PAH, into aromatized products. Nowadays, LSF is being used in the following forms:

- Liquids for spraying, nebulization, immersion, or showering
- Emulsions incorporated into foods by injection or mixing
- Water-mixable emulsions for showering or curing brine
- Powders such as maltodextrins, salt, saccharides, starch, proteins, and seasonings
- Solutions in vegetable oils (Borys, 2004).

Polycyclic Aromatic Hydrocarbons (PAH)

Apart from the compounds mentioned above, there are also conditions suitable for formation of other compounds during a smoke production. One of the most important groups which are actually harmful to human health is the group of PAH. These are being formed during the thermal decomposition of wood, especially at limited access of oxygen in the range of 500–900°C (Bartle, 1991). PAH are characterized by two or more condensed aromatic rings in a molecular structure and have a strong lipophilic character. The temperature of smoke generation plays a decisive role because the amounts of PAH contained in smoke increase linearly with the temperature of smoke generation in the interval of 400–1000°C (Tóth & Blaas, 1972). Apart from the formation itself, the temperature also affects the structure and the number of PAH. The number of PAH present in smoked fish can reach the value of 100 various compounds (Grimmer & Böhnke, 1975).

Behavior of PAH in an Organism

According to current knowledge, some PAH are able to interact with enzymes (such as aryl hydrocarbon hydroxylases) in organisms to form PAH dihydrodiol derivates. These reactive products (so-called "bay region" dihydrodiol epoxides) are believed as ultimate carcinogens that are able to form covalently bounded adducts with proteins and nucleic acids. In general, DNA adducts are thought to initiate cell mutation which results in malignancy (Bartle, 1991). A direct mutagenic potential of 14 PAH and PAH, containing fractions isolated from smoked and charcoal broiled samples, was studied toward strains TA 98 and TA 100 using the Ames test. The most potential mutagenicity was observed on PAH fractions isolated from smoked fish, treated before smoking with nitrites in an acid solution (Kangsadalampai, Butryee, & Manoonphol, 1997).

To simplify an interpretation of real risk of PAH to human health, there have been attempts to express objectively the real risk using toxic equivalency factors (Nisbet & La Goy, 1992). However, this approach does not reflect wider aspects of a potential toxicity of oxidized PAH products due to the effect of ultraviolet light, as well as other environmental factors (Law, Kelly, Baker, Jones, & McIntosh, 2002). Moreover, PAH content in smoked foods can be affected not only by environmental factors, but also by diffusion processes into plastic packaging materials (Šimko, 2005).

Legislative Aspects and International Normalization of PAH in Smoked Meat and Liquid Smoke Flavor

With regard to the harmful effects of PAH on living organisms, some European countries had enacted maximum limits of these compounds in smoked meat products at different levels in the past. To simplify suchlike problems associated with the variability of PAH composition, benzo[*a*]pyrene (BaP) had been accepted as the indicator of total PAH presence in smoked foods, even in spite of the fact that BaP constitutes only between 1 and 20% of the total carcinogenic PAH (Andelman & Suess, 1970). At present time, the situation in EU has unified by adoption of the EC Regulation 1881/2006 limiting BaP content at level of 5 μg kg^{-1} in smoked meats, smoked meat products, muscle meat of smoked fish, and smoked fishery products. Apart from this, EC has also adopted either the directive 2005/10/EC laying down the sampling methods and the methods of analysis for the official control of BaP levels in foodstuffs or the recommendation 2005/108/EC on the further investigation into the levels of PAH in certain foods such as benzo[*a*]anthracene BaA), benzo[*b*]fluoranthene (BbF), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), benzo[*g,h,i*]-perylene (BghiP), chrysene (Chr), BaP, cyclopenta[*c,d]*pyrene (CcpP), dibenzo[*a,h*]anthracene (DahA), dibenzo[*a,e*]pyrene (DaeP), dibenzo[*a,h*]pyrene (DahP), dibenzo[*a,i*]pyrene (DaiP), dibenzo[*a,l*]pyrene (DalP), indeno[1,2,3-*cd*]pyrene (I*cd*P), and 5-methylchrysene. Joint expert committee for contaminants and additives, FAO and WHO (JECFA), has defined another compound benzo[*c*]fluorene (BcF), which should also be monitored with regard to its effects to living organisms. Concerning LSF, the EC has adopted the regulation 2065/2003 relating to the production of smoke flavorings intended to be used for food flavoring. This regulation has limited the maximum acceptable concentrations of BaP at 10 μg kg^{-1} and BaA at 20 μg kg^{-1} in these products. Finally, the directive 88/388/EEC has limited the maximum residual levels of BaP at 0.03 μg kg^{-1} in foodstuffs flavored by LSF. For international trade purposes, Joint Expert Committee for Food Additives and Contaminants of FAO and WHO has adopted a specification, which tolerates the concentration in liquid smoke flavors (LSF) at the levels of 10 μg kg^{-1} for BaP, and 20 μg kg^{-1} for BaA, respectively (Report of the Joint FAO/WHO Expert Commission, 1987).

Analysis of PAH

Due to the fact that PAH are presented in food at the μg kg^{-1} levels, algorithm of the analysis is usually composed from such steps as extraction/hydrolysis of food matrix, liquid/liquid partition, cleanup procedures, concentration, chromatographic separation and, of course, determination. Although all steps are very important, anyway, chromatographic separation is the most important for correct evaluation of real risk assessment, for example, while BaP is a very strong carcinogenic agent, carcinogenic activity of its isomer benzo[*e*]pyrene is quite low. Methodology of PAH analysis was strongly affected by levels of development of chromatographic methods themselves. In the middle of last century, separation of BaP isomers by paper and column chromatography had been practically impossible (Schaad, 1970). With regard to complex mixtures of PAH, the presence of varying interfering substances and the need to correctly assess real concentrations of the most dangerous compounds at minimum, it was necessary to overcome problems regarding a resolution of so-called "benzopyrene fraction" which consisted at this time from BaP and its isomer BeP, BkF, BbF, and perylene (Per). In 1968, at a join meeting of Indiana University Cancer Center and International Agency for Research on Cancer it had been specified that any acceptable analytical method should be capable of separating at least BaA, BaP, BeP, BghiP, pyrene (Py), BkF, and Cor (Howard & Fazio, 1980). Collaborative studies of a method specific for BaP and a general procedure for PAH were conducted under the auspices of the Association of Official Analytical Methods (AOAC) and International Union of Pure and Applied Chemistry (IUPAC). Procedures consisted of an initial saponification of the sample in ethanolic potassium hydroxide solution, followed by a partition step between dimethylsulfoxide (DMSO) and an aliphatic solvent followed by column chromatography on pre-treated Florisil. For determination of individual PAH, a cellulose reverse phase technique in conjunction with cellulose acetate multiphase technique was used. The method was adopted as an AOAC official first action method in 1973 and accepted as a recommended method by IUPAC. Statistical evaluation of the data obtained by interlaboratory tests, in which ham samples were fortified with BaP, BeP, BaA, and BghiP at a level of 10 μg kg^{-1} and analyzed by above mentioned method showed standard deviation between 7.4 and 12.7%. On this basis, the method has been adopted as official method of AOAC (AOAC, 1995).

Sample Preparation

Smoked meat and LSF represent two different matrices, which share only organoleptic profile and compounds to be determined. For this reason, different procedures for sample pre-treatment are developed to reach as highest recoveries of analytes as possible.

Sample Treatment of Smoked Meat

From analytical point of view, meat and its products belong to problematic matrices with regard to the presence of various interfering compounds. Moreover, PAH as lipophile compounds have tendency to diffuse not only into non-polar part of the sample but also inside of tissue cells due to existing concentration gradient. For this reason a simple solvent extraction with non-polar solvent seems to be insufficient to reach high recovery. Grimmer and Böhnke (1975) isolated PAH from smoked fish and smoked-dried cobra with boiling methanol prior to sample hydrolysis with methanolic KOH. It was found that only about 30% BaP and other PAH was extractable from the samples, whereas an additional alkaline hydrolysis of meat protein yielded another 60% of PAH. It was concluded that PAH were linked adsorptively to high molecular structures not destroyed with boiling methanol. Although more than 80% of the methanol used could be recovered, this contained only one-third of the PAH contained in the sample. As postulated, alkaline hydrolysis with aqueous methanolic KOH is an absolute necessity to isolate PAH quantitatively from such types of samples. Alkaline hydrolysis takes usually 2–4 h depending on character of sample. Lean tissues take less time as adipose and collagen-containing tissues. This sample treatment was adopted followingly in many experimental works (Fretheim, 1976; Binnemann, 1979; Larsson, 1982; Lawrence & Weber, 1984). On the other hand, Vassilaros, Stoker, Booth, and Lee (1982) observed that the use of an alcohol is superfluous and contributes to interference problems in the final analysis because of methyl esters formation from fatty acids and methanol which are then difficult to remove from PAH fraction. Takatsuki, Suzuki, Sato, and Ushizawa (1985) found that during alkaline hydrolysis BaP may be partially decomposed by the coexistence of alkaline conditions, light oxygen, and peroxides in aged ethyl ether. They proposed to use amber glass, addition of Na_2S as antioxidant, distillation ethyl ether just before use and prevention of air from contact with adsorbents. To protect PAH from light decomposition, Karl and Leinemann (1996) used brown glassware carefully rinsed with acetone before alkaline hydrolysis, even though some authors also recommended direct extraction with organic solvents. Potthast and Eigner (1975) proposed a procedure based on mixing of pre-ground sample with chloroform and anhydrous Na_2SO_4 to remove water from extract. After adding Celite, the fat portion became uniformly distributed over the surface of the adsorbent. Although authors achieved a recovery 95–100% of BaP added at level of 10 μg, there is a real assumption that they recovered only "free" PAH accessible with solvent. This procedure was used also in the work of Alonge (1988). Cejpek, Hajšlová, Jehličková, and Merhaut (1995) tested efficiency of several organic solvents to obtain fat portion from meat samples. The best efficient solvent was mixture of chloroform–methanol (2:1), less effective was chloroform and the worst yields were achieved with methanol. This confirms observations of Grimmer and Böhnke (1975) regarding insufficient capability of methanol to extract quantitatively PAH from meat samples. Otherwise, the use of chloroform–methanol

mixture, also called Folch agent, is widely used in food analysis for extraction of lipids, when methanol makes possible extraction of lipids from inside of cells by denaturation of cell wall proteins. Joe, Salemme, and Fazio (1984) digested samples of smoked food with KOH, and extracted PAH with Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane). Chen, Wang, and Chiu (1996) compared efficiency of extraction from freeze-dried sample, when sonication and Soxhlet procedures were employed. Recovery studies showed that Soxhlet extraction was mere suitable prior to the sonication method. Accelerated procedure of extraction was tested by Wang, Lee, Lewis, Kamath, and Archer (1999). Samples were extracted in a Dionex extractor as well as Soxhlet apparatus. Advanced solvent extraction technique (ASE) was found to be comparable with, even better than reference Soxhlet method, when significant reductions in time of extraction and solvent consumption were achieved. García-Falcón, Simal-Gandara, and Carril-Gonzalez-Barros (2000) accelerated extraction of PAH from freeze-dried samples into hexane with microwave treatment and hexane extract than saponified with ethanolic KOH.

Sample Treatment of Liquid Smoke Flavors

Sample treatment of LSF matrix is different from the treatment of processed meats due to easy access of organic solvent "inside" a liquid matrix. For this, there is not usually any reason to treat samples by time-consuming hydrolysis under reflux. Different situation could have arisen, when LSF are in solid state (e.g., applied on starch, gelatine, or encapsulated). In spite of this, some authors preferred alkaline hydrolysis of LSF under reflux. However, adding of KOH is strongly recommended to transform phenols to polar, non-extractable phenolates prior to the PAH extraction with non-polar solvent. White, Howard, and Barnes (1971) alkalized water-soluble LSF and resinous condensates which settled out of LSF after storage with KOH solution and extracted PAH into isooctane. Silvester (1980) extracted PAH from alkalized liquid SFA with hexane. Radecki, Lamparczyk, Grzybowski, and Halkiewicz (1978) alkalized LSF with ethanolic KOH solution and maintained it at 60°C for 30 min prior to the extraction into cyclohexane. After alkalization, a direct extraction of PAH with cyclohexane was used by Šimko, Petrík, and Karovičová (1992). On the other hand, Gomaa, Gray, Rabie, Lopez-Bote, and Booren (1993) saponified liquid LSF with methanolic KOH for 3 h and then extracted PAH into cyclohexane. Laffon Lage, Garcia Falcon, Gonzalez Amigo, Lage Yusty, and Simal Lozano (1997) used solid phase extraction (SPE) technique on Sep Pak C18 for PAH isolation and compared to (supercritical fluid extraction) SFE procedure, when the sample for SFE was mixed with alumina and extracted PAH were concentrated in octadecylsilane trap. In both cases, 91% recoveries of BaP spiked at 15 ng were found and statistically no significant differences were observed. Taking into account expensive SFE extractor, they recommended using simple SPE procedure. Guillén, Sopelana, and Partearroyo (2000a, 2000b) alkalized LSF with methanolic KOH and heated under reflux for 3 h, with following extraction of PAH into dichloromethane, or cyclohexane, respectively.

Pre-Separation Procedures

At this point, both procedures are more or less the same for processed meats and LSF. But sometimes, mainly after adipose tissue hydrolysis, the presence of lipoproteins in non-polar solvent needs their removal prior to pre-separation with one-step liquid–liquid partition between non-polar and polar solvents (e.g., hexane–water/dimethylformamid (Grimmer & Böhnke, 1975), methanol/water, or dimethylsulfoxide/water–cyclohexane (Lawrence & Weber, 1984; Karl & Leinemann, 1996)), or two-step liquid–liquid partition (e.g., NaCl/water and DMF/water (Vaessen, Jekel, & Wilbers, 1988)), or precipitation of lipoproteins with Na_2WO_4(Šimko, 1991; Šimko, Gombita, et al., 1991; Šimko, Karovičová, & Kubincová, 1991; Šimko, Gergely, Karovičová, Drdák, & Knežo, 1993). For pre-separation, deactivated Florisil (Šimko, Karovičová, et al., 1991; Lawrence & Weber, 1984; Wang et al., 1999; Gomaa et al., 1993; Guillén et al., 2000a; Šimko et al., 1993; Mottier, Parisod, & Turesky, 2000; Stijve & Hischenhuber, 1987), silica gel (Larsson, 1982; Takatsuki et al., 1985; Mottier et al., 2000), alumina (Vaessen et al., 1988), and Celite (White et al., 1971; Silvester, 1980) are used frequently. The only study (Silvester, 1980) reported that elution of BaP from Florisil and silica gel with hexane was impossible and for this reason alumina was recommended for pre-separation of concentrated PAH extracts. Guillén et al. (2000b) preferred elution of silica with cyclohexane prior to Florisil dichloromethane elution to obtain higher recoveries with reduced amounts of interfering substances, which were eluted from Florisil with dichloromethane. Effective pre-separation procedure is also GPC (gel permeation chromatography) on Sephadex LH 20 (Takatsuki et al., 1985) or BioBeads S-X3 (Cejpek et al., 1995), respectively. Mottier et al. (2000) cleaned concentrated cyclohexane extracts by SPE, using conditioned Isolute aminopropyl and C_{18} columns. Also, the usage of two different techniques was applied, when cyclohexane extract was first cleaned with GPC on Sephadex LH 20 and followingly on silica gel (Vaessen et al., 1988), eventhough the last procedure is also possible to be carried out in reverse mode (Afolabi et al., 1983). In all cases, removal of organic solvents by vacuum evaporation to concentrate PAH is an unavoidable operation. This may be a critical step, mainly if there is a presumption of presence of light PAH as fluorene (Flu), antracene (Ant), and phenanthrene (Phe) in the extracts. In this case, organic solvents should not be evaporated to dryness because these PAH could be lost due to their volatility. This cautious manipulation is not necessary, if only PAH with boiling point above 370°C are determined (Grimmer & Böhnke, 1975).

Thin Layer Chromatography

Thin layer chromatography (TLC) belongs to older analytical methods to be used for determination of PAH in various matrices. Haenni (1968) discussed the development of analytical tools for control of PAH in food additives and in

food by the use of ultraviolet specification within specific wavelength ranges. To this, Schaad (1970) reviewed various chromatographic separation procedures, including TLC. White et al. (1971) used two systems for PAH separation. The first consisted of 20% *N*,*N*-dimethylformamide in ethyl ether as stationary phase and isooctane as mobile phase. Fluorescent spots were scraped out from cellulose layer and eluted with hot methanol. After concentration, the sample was developed in second system, using ethanol–toluene–water (17:4:4) as developer. Fluorescent spots were eluted again from cellulose acetate layer and ultraviolet spectrum was recorded against isooctane in a reference cell. The observed maxima were compared with those in the spectra of known PAH obtained under the same instrumental conditions. Estimation of quantity of the identified compounds was made by the baseline technique in conjunction with spectra of these PAH and the identification was confirmed by spectrophotofluorometry. This method has become a base of AOAC Official Method 973.30, adopted in 1974.

Gas Chromatography

Nowadays, gas chromatography (GC) is widely used for determination of PAH in food analysis. The determination of the large number of PAH in samples requires columns with high efficiency. To separate some critical pairs as well as isomers of methyl derivatives of certain PAH, capillary columns (50 m $\times$ 0.3–0.5 mm) which can achieve 50,000–70,000 high equivalent theoretical plate (HETP) are especially convenient. However, packed columns used for determination of PAH (Grimmer & Böhnke, 1975) had lower HETP ranging between 20,000 and 30,000 and for this reason were not suitable for quantity determination. Two stationary phases, OV-17 and OV-101 were used for separation of BaP from BeP, DajA from DahA, and Phe from Ant. Successful separation of Chr from BaA was achieved using OV-17 stationary phase, but separation of BbF, BjF, and BkF isomers on packed columns was not possible (Grimmer & Böhnke, 1975). Radecki et al. (1978) tested various stationary phases (GE SE 30; OV-1; SE-52; OV-7; OV-101; BMBT; BBBT) on Chromosorb W, Chromosorb W HP, Gas Chrom, and Diatomite CQ supports in packed columns to develop a precise GC method for assaying BaP in LSF. However, separation of BaP from BeP and Per was not possible to achieve using SE 30, OV-1, SE-52, OV-7, and OV-101 stationary phases. Nematic phases gave a good separation of BaP from its isomers, but they were not suitable for analysis with regard to their poor thermal stability. Detection of PAH is not a serious problem, because a response of flame ionization detector (FID) is practically equal for all compounds and is linear over a large concentration range (about $1–1\times10^6$), according to the carbon content. However, the use of FID is sometimes hampered by the need for very thorough cleanup procedures with accompanying risk of severe losses and possible misidentification (Tuominen, Wickström, & Pyysalo, 1986). Mass spectrometry detector (MSD) has also been used successfully for PAH analysis in many cases (Lee, Novotny, & Bartle, 1981). Especially, the use of MSD operating in selected ion

monitoring mode makes possible to simplify the time-consuming cleanup procedure (Tuominen et al., 1986) and it is recommended especially for quantitative analysis. Ion trap detector (ITD) has some advantages prior to traditional MSD. The ITD utilizes electric fields to hold the ions within the ion storage regions. The ITD is then scanned through the mass range, causing the ions to be ejected from this region sequentially, from low to high mass. The ejected ions are detected by a conventional electron multiplier. Thus the characteristic of the ITD is that ionization and mass analysis take place in the same space. This contrasts with a conventional MSD, which requires a separate ionization source, focusing lenses and analyzer, which is associated with low mechanical tolerances (Williams, Andrews, Bartle, Bishop, & Watkins, 1988). Sometimes, separation of isomers is a quite serious problem even though capillary columns are used. Dennis et al. (1984) were not able to separate BjF from BkF. Speer, Steeg, Horstmann, Kuehn, and Montag (1990) were not able to separate Chr from triphenylene (Tph), BbF, BjF, and BkF from each other and DahA from DacA. Problems associated with separation of Chr from Tph are also reported in works of Guillén et al. (2000a, 2000b). Wise, Sander, and May (1993) informed about difficulties to separate isomers BbF and BkF. On the other hand, Chen and Chen (2005) separated BbF and BkF sufficiently on DB-1 fused silica capillary column. Jira (2004) separated all 16 European priority PAH compounds including problematic BbF, BjF, and BkF isomers using GC column VF 17 ms. Review of pre-separation procedures as well as GC conditions to be used for determination of PAH in smoked meat products and LSF are summarized in Table 13.1.

High Pressure Liquid Chromatography

In recent years, the high pressure liquid chromatography (HPLC) method has been used intensively for determination of PAH in food, as reported in review works (Bartle, 1991; Tamakawa, 2004; Stahl & Eisenbrand, 1988). Formerly used stationary phases, such as alumina and silica gel, were later replaced with chemically bonded phases, particularly reverse phases such as ODS, widely used at present time. For determination of PAH in food, Hunt, Wild, and Crosby (1977) developed phtalimidopropylsilane (PPS) stationary phase and compared it with octadecylsilane (ODS). As found, PPS column was able to separate BkF from Per, which was impossible by ODS column. HPLC has some advantages in PAH analysis as follows (Tamakawa, 2004):

- Separation of isomers shows very good resolution
- Sufficient sensitivity and specificity of ultraviolet fluorescence detection
- Molecular sizes of PAH can be estimated on the basis of retention time using reversed phase column
- Possibility to determine compounds with high molecular weight
- Analyses are usually carried out at ambient temperature; there is no risk of thermal decomposition of analytes.

Table 13.1 Pre-separation procedures and GC conditions to be used for determination of PAH in smoked meat products and liquid smoke flavors

Sample	Sample treatment and pre-separation	Column/stationary phase	Temperature program	Detection	References
Barbecued sausages	Saponification with mixture of ethanol, water, and KOH, extraction with cyclohexane, pre-separation by SPE on Isolute aminopropyl and C_{18} columns	25 m × 0.2 mm capillary column/ SPB–5	80°C for 0.5 min → 230°C at 8°C/min → 300°C at 5°C/min	MSD	Mottier et al. (2000)
Smoked fish	Extraction with pentane, pre-cleaning on silica gel and Sephadex LH-20	25 m × 0.2 mm quartz capillary column/SE-54	100 → 260°C, 3°C/min	MSD	Afolabi et al. (1983)
Smoked fish	Saponification in methanolic KOH, liquid–liquid extraction (methanol–water–cyclohexane and DMF–water–cyclohexane) and GPC on Sephadex LH 20	10 m × 2 mm packed columns/5% OV-101 and OV-17 on sorbent Gas Chrom	1. 120 → 250°C, 1°C/min 2. 250°C, isothermal	FID MSD	Grimmer and Böhnke (1975)
Smoked sausages	Saponification in methanolic KOH, liquid–liquid extraction (methanol–water–cyclohexane and DMF–water–cyclohexane) precleaning on silica gel and GPC on Sephadex LH 20	10 m × 2 mm packed column/5% OV-101 on sorbent Gas Chrom	260°C isothermal	FID	Fretheim (1976)
Smoked meat products	Saponification with mixture of methanol, water, and KOH, partition with DMF, precleaning on Kieselgel 60	25 m × 0.28 mm capillary column/SE-54	240°C isothermal	MSD	Binnemann (1979)
Smoked fish and fish products	Saponification in methanolic KOH, liquid–liquid extraction (methanol–water–cyclohexane and DMF–water–cyclohexane) precleaning by CC on silica gel and GPC on Sephadex LH 20	55 m × 0.3 mm glass capillary column/ SE-54	165°C for 6 min, 165 → 255°C, at 4°C/ min	FID	Larsson (1982)
Smoked fish, smoked meat spreads	Saponification with mixture of methanol, water, and KOH, extraction with cyclohexane, cleaning up on Florisil, partitioning with DMSO/hexane	30 m × 0.25 mm capillary column/ DB-5	25°C, → 180°C rapidly → 320°C at 8°C/ min	FID, MSD	Lawrence and Weber (1984)

Table 13.1 (continued)

Sample	Sample treatment and pre-separation	Column/stationary phase	Temperature program	Detection	References
Smoked fish	Saponification with methanol–water–KOH mixture under reflux, extraction into cyclohexane, extraction of PAHs with caffeine/formic acid, washing with NaCl solution, extraction into cyclohexane, pre-separation on silica gel	30 m × 0.25 mm capillary fused silica column/DB-5	110°C isothermal for 1.5 min → 210°C at 30°C/min → 290°C at 3°C/min → 300°C at 10°C/min	MSD	Karl and Leinemann (1996)
Smoked salmon, sausages, pork	Direct solvent extraction (ASE), cleanup on Florisil	30 m × 0.25 mm capillary column/cross linked 5% phenyl methyl siloxane HP-5MS	40°C isothermal for 1 min → 250°C at 12°C/min → 310°C at 5°C/min	MSD	Wang et al. (1999)
LSF	Alkalisation with KOH solution, extraction with cyclohexane, cleanup on silica	25 m × 0.2 mm fused silica capillary column/HP-5 cross linked with 5% phenylmethylsilicone	50°C isothermal for 0.5 min → 180°C at 30°C/min → 300°C at 7°C/min	MSD	Šimko et al. (1992)
LSF	Heating with methanolic KOH under reflux, extraction with cyclohexane, cleaning up by SPE technique on Florisil	60 m × 0.25 mm fused silica capillary column/HP-5MS, 5% phenyl methyl siloxane	50°C isothermal for 0.5 min → 130°C at 8°C/min → 290°C at 5°C/min	MSD	Guillén et al. (2000a)
LSF	Heating with methanolic KOH under reflux, extraction with cyclohexane, cleaning up by SPE technique on LC silica	60 m × 0.25 mm fused silica capillary column/HP-5MS, 5% phenyl methyl siloxane	50°C isothermal for 0.5 min → 130°C at 8°C/min → 290°C at 5°C/min	MSD	Guillén et al. (2000b)
Smoked meats	Saponification with methanolic KOH, extraction with cyclohexane, partition with DMF/water, cleanup on silica gel and with GPC on Bio Beads S-X3	50 m capillary column/DB-5	70°C → 280°C at 5°C/min	MSD	Speer et al. (1990)
Smoked chicken	Extraction with methanol in Soxhlet app. + KOH, extraction into n-hexane, cleanup on Sep-Pak Florisil cartridge	30 m × 0.32 mm/DB-5	70°C isothermal for 1 min → 150°C at 10°C/min → 280°C at 4°C/min hold for 14 min	ITD	Chiu et al. (1997)

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HPLC equipped with MSD is an effective tool for characterization of high molecular, thermally unstable compounds, e.g., BaP metabolites were identified and determined by this method in microbore mode (Bieri & Greaves, 1987). Due to high absorption of the light in UV part of spectrum and intensive fluorescence, both types of detectors are able to detect reliable concentrations at the μg kg^{-1} levels. On the other hand, measurements by non-specific detection systems, particularly optical detectors, though often precise, can also be much less accurate due to possible chemical interferences not having been chromatographically resolved or otherwise avoided prior to the measurement. The major impurities in the PAH fractions appear to be alkylated PAH, which have very similar responses in optical detection systems to their unsubstituted analogues (Sim et al., 1987). Regarding diode array detector, confirmation of peak purity and identification is possible, but due to the broad absorption bands in UV spectra it is highly probable that there will be some interference, if one particular wavelength is chosen for quantification. In any way, identification must be based on retention time. Fluorescence detector provides very high selectivity and sensitivity, particularly those with excitation and emission wavelengths that can be varied throughout the analyses. However, fluorescence suffers from not being able to provide "broad spectrum" analyses (i.e., a wide variety of compounds) because of the presence of alkylated PAH compounds. Review of pre-separation procedures as well as HPLC conditions to be used for determination of PAH in smoked meat products and SFA are summarized in Table 13.2.

Comparison of Gas Chromatography and High Pressure Liquid Chromatography

In many works, also mentioned here, authors studied the advantages and drawbacks of both the methods, when studies were aimed especially at recovery studies, quality of separation processes, time of analysis, price of equipments, etc. Dennis et al. (1984) compared results of analysis of some food (two smoked) obtained by GC and HPLC. Thirty-five pairs of analysis were tested using statistical procedure (Student *t*-test). From this, 25 were not significantly different within the 95% confidence limits employed. But, data for BkF/benzofluorantenes and DahA/dibenzoanthracenes were not compared because different analytes were measured. Standard deviations indicated that repeatability of both methods was very good, being usually within 10% and gave comparable data throughout the wide range (0.2–1000 μg kg^{-1}). In conclusion of this study it was stressed that capillary GC possessed a much greater resolving power, in terms of plate number, so that many more PAH can be separated and determined. To the opposite, HPLC was able to separate individual isomers (BbF and BkF; Chr and Tph), i.e., it had a greater selectivity. Chiu, Lin, and Chen (1997) compared separation and detection conditions of both the

Table 13.2 Pre-separation procedures and HPLC conditions to be used for determination of PAH in smoked meat products and liquid smoke flavors

Sample	Sample treatment and pre-separation	Column/stationary phase	Mobile phase	Detection	References
LSF, smoked meats	Saponification with ethanolic KOH, extraction into cyclohexane, washing with saturate NaCl solution, cleanup on silica gel	25 cm × 4 mm Lichrosorb RP-18	Acetonitrile/water 8:2, isocratic, 1.5 ml min^{-1}	FLD Ex: 305, 381 nm Em: 389, 430, 520 nm	Alonge (1988)
Smoked meat products	Saponification with mixture of methanol, water and KOH, extraction with cyclohexane, washing with Na_2WO_4 solution, cleanup on Florisil	30 cm × 3 mm, Separon SGX C_{18} RP-5 μm	Acetonitrile/water 3:1, isocratic, 1.5 ml min^{-1}	FLD Ex/Em 310/410 nm	Šimko (1991), Šimko et al. (1991, 1992, 1993)
Smoked fish, smoked meat spreads	Saponification with mixture of methanol, water and KOH, extraction with cyclohexane, cleaning up on Florisil, partitioning with DMSO/hexane	25 cm × 4.6 mm, RP-18, 5 μm	Acetonitrile/water 7:3, isocratic, 3 ml min^{-1}	UVD 254 nm FLD Ex/Em 250/370 nm	Lawrence and Weber (1984)
Fish, shellfish	Saponification with methanol–water–KOH mixture under reflux, extraction into n-hexane, cleanup on silica gel	Radial-Pak PAH	Acetonitrile/water 8:2, isocratic, 1 ml min^{-1}	FLD Ex/Em 370/410 nm	Vassilaros et al. (1982)
Smoked fish	Saponification with methanol–water–KOH mixture under reflux, extraction into cyclohexane, extraction of PAHs with caffeine/formic acid, washing with NaCl solution, extraction into cyclohexane, pre-separation on silica gel	ET 15 cm × 4 mm, Nucleosil 5 C_{10} PAH	Acetonitrile/water 7:3 for 1 min than gradient linearly up to 9:1 in 19th min, than to 100% acetonitrile from 20 to 40 min than isocratic till 55 min	UVD 240, 254, 260 nm FLD Ex/Em 300/408 and 280/395 nm	Karl and Leinemann (1996)
Smoked sausage, smoked meat	Extraction with chloroform/methanol mixture, pre-separation by GPC on Bio Beads S-X3	15 cm × 4.6 mm Supelcosil LC PAH, 5 μm	A: methanol/acetonitrile/water 50:25:25 B: acetonitrile; 1 min 100% A, 25th min. 100% B	FLD Variable Ex (240–293) Em (340–498) nm	Cejpek et al. (1995)

Table 13.2 (continued)

Sample	Sample treatment and pre-separation	Column/stationary phase	Mobile phase	Detection	References
Smoked frankfurters, smoked meats	Extraction with methanol in Soxhlet app. + KOH, extraction into n-hexane, cleanup on Pep-Pak Florisil	12.5 cm × 4.6 mm Envirosep-pp C_{18} 5 μm	I. Acetonitrile/water 7:3, isocratic, 2 ml min^{-1} II. Acetonitrile/water 40:60, gradient to 100% acetonitrile in within 25 min III. Acetonitrile/water 55:45, gradient to 100% acetonitrile in within 23 min	UVD 230–360 nm FLD Variable Ex (232–302) Em (330–484) nm	Chen et al. (1996)
LSF	Alkalisation with NaOH solution, extraction with hexane, cleanup on alumina	25 cm × 4.6 mm Partisil 10 ODS	Methanol/acetonitrile/water 35:35:30, isocratic	FLD Ex/Em 280/390 nm	Silvester (1980)
LSF	Alkalisation with ethanolic and aqueous NaOH, extraction into cyclohexane, partitioning with DMSO/water, extraction into cyclohexane	30 cm × 4 mm, μBondapak C_{18}/Corasil	Methanol/water 7:3, 2 ml min^{-1}	UVD 280 nm	Radecki et al. (1978)
LSF, smoked food products	LSF: Saponification with methanolic KOH, extraction into cyclohexane, purification on Florisil Meat products: digestion with KOH solution, extraction with Freon 113, purification on Florisil	25 cm × 4.6 mm, Supelcosil LC-PAH	Acetonitrile/water 60:40 for 5 min than 100% of acetonitrile in 15 min, hold for 15 min than decrease to 60% during 10 min	FLD Ex/Em 254/375 nm	Gomaa et al. (1993)
Smoked fish	Direct extraction with chloroform, pre-separation on preparation silica column	Preparation column: 25 cm × 4.6 mm, silica 5 μ Analytical column: 15 ×4.6 mm 5 μ particle, Supelcosil LC-PAH	Preparation column: pentane/5% DCM, 0.8 ml min^{-1} Analytical column: water/acetonitrile 6:4 for 5 min than to 100% acetonitrile over 40 min, 1.5 ml min^{-1}	FLD Variable nm	Moret, Conte, and Dean (1999)
Smoked fish, ham	Saponification with mixture of methanol, water, and KOH, extraction with cyclohexane, partitioning with DMSO/hexane	Spherisorb ODS 5 μm precolumn and 5 μm VydacODS analytical column	Acetonitrile/water 6:4, linearly to 9:1 during 35 min	FLD Ex/Em 290/430 nm	Dennis et al. (1984)
Smoked meats products	Saponification with methanolic KOH, extraction with n-hexane, pre-separation by SPE on CN bonded silica	Nucleosil 100–5 C 18 PAK	Acetonitrile/water 8:2, isocratic, 0.5 ml min^{-1}	FLD Ex/Em 290/430 nm	Hartmann (2000)

Table 13.2 (continued)

Sample	Sample treatment and pre-separation	Column/stationary phase	Mobile phase	Detection	References
Smoked fish	Saponification with methanol–water–KOH mixture under reflux, extraction into n-hexane, cleanup on silica gel	15 cm × 6 mm, ODS, 5 μm particles	Acetonitrile/water 8:2, isocratic, 1 ml min^{-1}	FLD Ex/Em 370/410 nm	Ova and Onaran (1998)
LSF, smoked foods	LSF: Saponification with methanolic KOH, extraction into cyclohexane, purification on Florisil Smoked products: digestion with KOH solution, extraction with Freon 113, purification on Florisil	12.5 cm × 4 mm Lichrosphere 100 RP-18	A: water; B: methanol/acetonitrile 1:1; I. segment: 1:80 to 100% B in 20 min II. segment: 100% B for 5 min III segment: 100 to 80 B in 5 min	FLD Ex/Em 365/418 nm	Yabiku, Martins, and Takahashi (1993)
Smoked meat products	Saponification with methanolic KOH, extraction into cyclohexane, pre-separation by SPE on Kiesel gel	12.5 cm × 4 mm, Chrompack PAH-Säule	Acetonitrile/water 9:1, isocratic, 0.5 ml min^{-1}	FLD Ex/Em 290/430 nm	Räuter (1997)
Smoked chicken	Extraction with methanol in Soxhlet app., + KOH, extraction into n-hexane, cleanup on Sep-Pak Florisil	12.5 cm × 4.6 mm Envirosep-pp 5 μm C_{18}	Acetonitrile/water 55:45, gradient to 100% acetonitrile in within 23 min 1.2 ml min^{-1}	FLD Variable nm	Chiu et al. (1997)

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methods analyzing smoked chicken. As found, 16 priority PAH pollutants set by Environmental Protection Agency (EPA) can be simultaneously separated by HPLC using a gradient solvent system and detection by FLD with seven setting of programmable wavelength. With GC, a temperature programming method makes it possible also to resolve these 16 PAH. The presence of impurities in smoked meat products can interfere with the identification and quantification of PAH by HPLC. With ITD, the PAH can be identified even in the presence of fat- or PAH-like impurities. The retention times by HPLC were shorter than those by GC when HPLC had a better separation for most compounds than GC. Sim et al. (1987) compared GC and HPLC methods analyzing 16 PAH pollutants. As pointed out, the chromatographic resolution may be divided into a combination of column capacity, column efficiency, and separation selectivity. GC has higher column efficiency and thus has an advantage for complex mixture analysis, but HPLC can often have higher column selectivity, which is more suitable for separation of isomeric compounds. Thus, both methods should be viewed as complementary in the analysis of PAH and they are essential for precise and reliable analysis.

Occurrence of PAH

Immediately after information regarding carcinogenic effect, research workers started to find the real situation of PAH content in smoked meat products. These data approve that technologically correct smoking process contaminates meat products only with small PAH content – usually below 1 $\mu g\ kg^{-1}$. Far more dangerous is smoking process at uncontrolled conditions, typical for home "wild" smoking in preparation of heavy smoked "farm" products as well as smoking being done in developing countries without any technological knowledge and hygienic control. These products bring a serious real risk to consumer to fall in cancer, especially after long time of consumption due to BaP content reaching sporadically eventhought up to several hundreds $\mu g\ kg^{-1}$ (Šimko, 2002; Dobríková & Světlíková, 2007).

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Chapter 14
Veterinary Drugs and Growth Promoters Residues in Meat and Processed Meats

Milagro Reig and Fidel Toldrá

Introduction

Veterinary drugs, which comprise a large number of different types of substances, are generally intended for therapeutic (to control infectious diseases) and prophylactic (to prevent against infections) purposes in farm animals. Other substances with growth promoting effect may exert antimicrobial effect against the microbial flora in the gut to take maximum profit of nutrients in the feed or by affecting the animal's metabolism. Most of these substances are orally active and can be administered either in the feed or in the drinking water. Other active hormones are applied in the form of small implants into the subcutaneous tissue of the ears. These are slow release (several weeks or months) devices and the ears are discarded at the slaughter. Growth promoters allow a better efficiency in the feed conversion rate. The net effect is an increased protein deposition, partly due to muscle proteases inhibition (Fiems, Buts, Boucque, Demeyer, & Cottyn, 1990), usually linked to fat utilization (Brockman & Laarveld, 1986). The result is a leaner meat (Lone, 1997) with some toughness derived from the production of connective tissue and collagen crosslinking (Miller, Judge, Diekman, Hudgens, & Aberle, 1989; Miller, Judge, & Schanbacher, 1990). Some recent fraudulent practices, consisting of the use of a kind of "cocktails" or mixtures of several substances like β-agonists and corticosteroids at very low amounts (Monsón et al., 2007), are difficult to detect with modern analytical instrumentation. They try to obtain a synergistic effect for a similar growth promotion with lower probability of detection by official control laboratories (Reig & Toldrá, 2007).

Thus, the use of veterinary drug substances as well as other substances with growth-promoting effects may usually constitute a clear economic benefit for farm production. However, its residues in the meat and other animal-derived

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Table 14.1 List of substances having anabolic effect (group A) and veterinary drugs (group B) in accordance to the Council Directive 96/23/EC (EC, 1996)

Group A: Substances having anabolic effect	Group B: Veterinary drugs and contaminants
1. Stilbenes	1. Antibacterial substances
2. Antithyroid agents	Sulfonamides and quinolones
3. Steroids	2. Other veterinary drugs
Androgens	(a) Antihelmintics
Gestagens	(b) Anticoccidials, including nitroimidazoles
Estrogens	(c) Carbamates and pyrethroids
4. Resorcyclic acid lactones	(d) Sedatives
5. Beta-agonists	(e) Nonsteroidal anti-inflammatory drugs
6. Other compounds	(f) Other pharmacologically active substances

foodstuffs may cause harmful effects to consumers when eating them, mainly depending on the type of substance and its content as residue in the foodstuff. Even though some studies with steroids have shown that when used properly, the amount of residues in the meat is minimal for any sensitive effect in comparison to natural steroid content (Lone, 1997), other substances may exert harmful effects on consumers.

This chapter is providing a summarized view of main veterinary drugs and growth-promoting substances that may be used legally or illegally in animal production. The main groups of these substances as listed in European Directive 96/23/EC (EC, 1996) are shown in Table 14.1. This will also briefly describe its properties, causes of concern, and measures for its control.

Main Groups of Substances with Anabolic Effect and Veterinary Drugs

Substances with Anabolic Effect

Steroid Hormones and Other Substances Having Hormonal Action

These substances exert estrogenic (except 17β-estradiol and ester-like derivatives), androgenic, or gestagenic action and may be used for growth-promoting purposes. These include steroid hormones and hormone-like substances and include testosterone, progesterone, trenbolone acetate, zeranol, and melengestrol acetate (see Table 14.2). Steroid hormones are essential for normal development and physiological function of most tissues. Synthetic hormones appear to bind to steroid receptors with equal or higher affinity than the natural hormones (Wilson, Lambright, Ostby, & Gray, 2002; Perry, Welshons, Bott, & Smith, 2005). So, trenbolone mainly binds to the androgen receptor, zeranol to the estrogen receptor and melengestrol that resembles natural progestins (EFSA, 2007). Maximum residue levels (MRL) have been established by national authorities and by the Codex Alimentarius. An important challenge

Table 14.2 Main properties of relevant androgens, estrogens, and gestagens

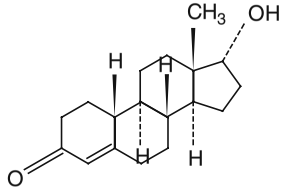
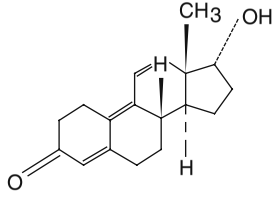
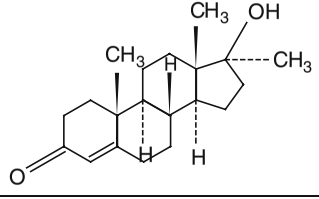
Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
ANDROGENS:							
17α-nortest-osterone	17-hydroxyestr-4-en-3-one	434-22-0		$C_{18}H_{26}O_2$	274.39	118	≈ Insoluble
17α-trenbolone	17α-hydroxyestra-4,9,11-trien-3-one	10161-33-8		$C_{18}H_{22}O_2$	270.38	186	Slightly soluble
17-methyltest-osterone	(17β)-hydroxy-17-methylandrost-4-en-3-one	58-18-4		$C_{20}H_{30}O_2$	302.44	161	≈ Insoluble

Table 14.2 (continued)

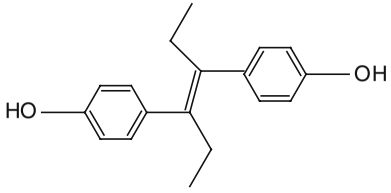
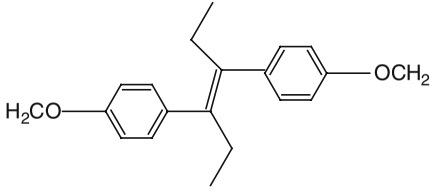
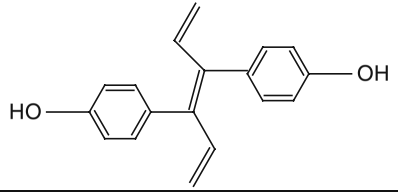
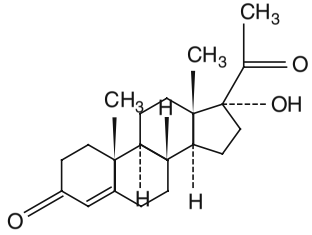
Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
ESTROGENS:							
Diethylstilbestrol	4,4'-(1,2-diethyl-1,2-ethene-diyl) bisphenol; α,α'-diethylstilbenediol	56-53-1		$C_{18}H_{20}O_2$	268.34	169	≈ Insoluble
Dimestrol	(E)-1,1'-(1,2-diethyl-1,2-ethenediyl) bis[4-methoxy benzene]; α,α'-diethyl-4,4'-dimethoxystilbene	130-79-0		$C_{20}H_{24}O_2$	296.39	124	≈ Insoluble
Dienestrol	4,4'-(1,2-diethylidene-1, 2-ethanediyl) bisphenol; 4,4'-(diethylidene ethylene)diphenol	84-17-3		$C_{18}H_{18}O_2$	266.32	227	≈ Insoluble

Table 14.2 (continued)

Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
GESTAGENS:							
17α - hydroxypro-gesterone	17-hydroxypregn-4-ene-3, 20-dione	68-96-2		$C_{21}H_{30}O_3$	330.45	222	≈ Insoluble

when analyzing these residues in meat is the ability to discriminate between endogenous production and exogenous administration.

Stilbenes

These substances are synthetic non-esteroidal estrogens. They exert estrogenic activity (growth and development of female sexual organs) and produce an increase of somatotropin secretion. Diethylstilbestrol was related to cancer and is banned. This substance leads to several reactive metabolites after oxidation in the body (Lone, 1997). Other stilbenes belonging to this group and its main properties are shown in Table 14.2.

Antithyroid Agents

These agents are able to interfere directly or indirectly on the synthesis, release or effect of the thyroideal hormones. These agents cause hypothyroidism by decreasing the basal metabolism rate, with water retention and weight increase. Representative compounds and its main properties are shown in Table 14.3.

Glucocorticoids

Corticoids are hormones of the adrenal cortex. These substances have physiological roles like the control of mineral and water balance. Glucocorticoids also have many important physiological functions and are thus involved in carbohydrate metabolism. They can be used with other hormones for growth promotion effect. Good representatives are dexamethasone and corticosterone. Main properties are reflected in Table 14.4.

β-Agonists

β-adrenergic agonists are used as therapeutic agents for respiratory disorders under prescription of veterinary inspectors. However, they have been extensively used as growth promoters because they bind to β receptors of various tissues and change the carcass composition. These substances reduce proteolysis and increase protein synthesis and lipolysis (Lone, 1997). This group includes numerous substances like clenbuterol, mabuterol, cimaterol, salbutamol, etc. Table 14.5 presents its main properties.

Antimicrobial and Antibiotic Drugs

Sulfonamides

This family of drugs is derived from sulfanilamide. Representative compounds are compiled in Table 14.6. They are broad spectrum antibiotics active against

Table 14.3 Main properties of relevant antithyroidal agents

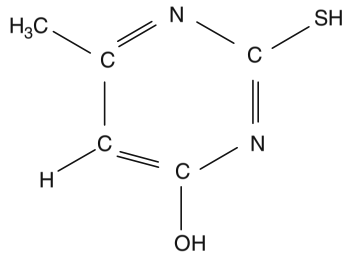
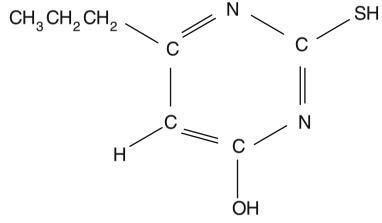
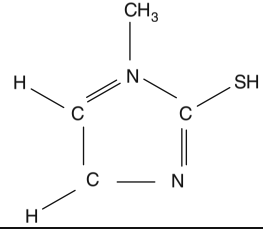
Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water (g/mL)
Methylthiouracil	2,3-dihydro-6-methyl-2-thioxo-4(1H)-pyrimidinone	56-04-2		$C_5H_6N_2OS$	142.18	326	Slightly soluble in warm water
Propylthiouracil	2,3-dihydro-6-propyl-2-thioxo-4(1H)-pyrimidinone	51-52-5		$C_7H_{10}N_2OS$	170.23	219	Slightly soluble
Tapazole	1,3-dihydro-1-methyl-2H-imidazole-2-thione	60-56-0		$C_4H_6N_2S$	114.17	146	Freely soluble

Table 14.3 (continued)

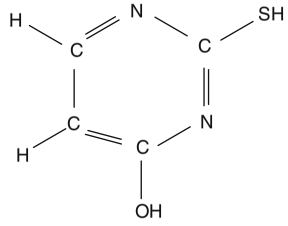
Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water (g/mL)
Thiouracil	2,3-dihydro-2-thioxo-4(1H)-pyrimidinone	141-90-2		$C_4H_4N_2OS$	128.15	No definite	Very slightly soluble

Table 14.4 Main properties of relevant glucocorticoids

Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Betamethasone	9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	378-44-9		$C_{22}H_{29}FO_5$	392.45	231	–
Dexamethasone	(11β,16α)-9-fluoro-11,17-21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	50-02-2		$C_{22}H_{29}FO_5$	392.45	268	Slightly soluble
Flumethasone	6,9-difluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	2135-17-3		$C_{22}H_{28}F_2O_5$	410.46	260	Insoluble

Table 14.4 (continued)

Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Corticosterone	(11β)-11,21-dihydroxypregn-4-ene-3,20-dione	50-22-6		$C_{21}H_{30}O_4$	346.45	180	Insoluble
Cortisone	17α,21-dihydroxy-4-pregnene-3,11,20-trione	53-06-5		$C_{21}H_{28}O_5$	360.46	220	Slightly soluble

Table 14.5 Names and main properties of representative agonists

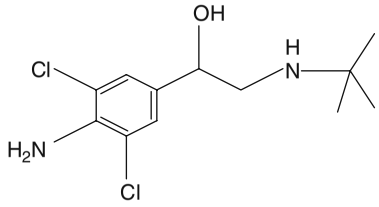
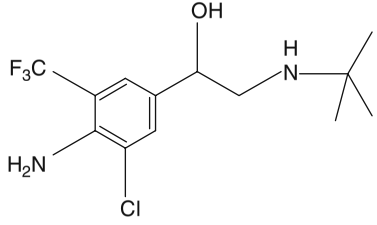
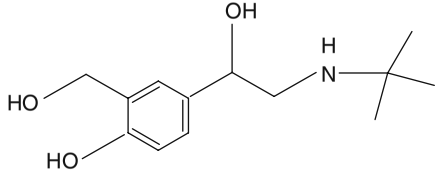
Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Clenbuterol	4-amino-α-[(tert-butilamino) methyl]-3,5-dichlorobenzyl alcohol	37148-27-9		$C_{12}H_{18}N_2Cl_2O$	277.19	174	Very soluble
Mabuterol	4-amino-3-chloro-α-[[(1,1-dimethyl-ethyl)amino]methyl]-5-trifluoromethyl) benzenemethanol	56341-08-3		$C_{13}H_{18}N_2F_3ClO$	310.75	205	Fairly soluble
Salbutamol	2-(hidroximetil)-4-[1-hidroxi-2-(tert-butilamino)etil]fenol	18559-94-9		$C_{13}H_{21}NO_3$	239.31	157–158	Sparingly soluble

Table 14.5 (continued)

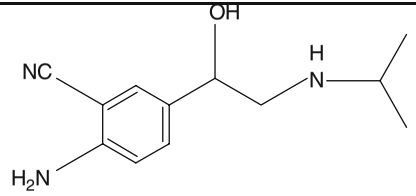
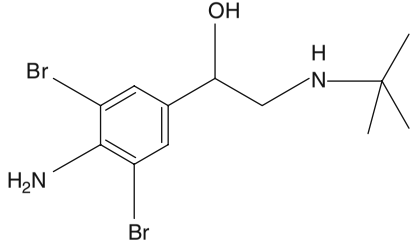
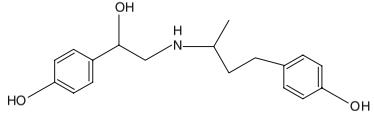
Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Cimaterol	2-amino-5-[1-hydroxy-2-[(1-methyl-ethyl) amino]ethyl] benzonitrile	54239-37-1		$C_{12}H_{17}N_3O$	219.29	159	Fairly soluble
Brombuterol	4-amino-a-[(*tert*-butylamino)methyl]-3,5-dibromobenzyl alcohol	41937-02-4		$C_{12}H_{18}Br_2N_2O$	366.08	–	Fairly soluble
Ractopamine	4-hydroxy-alpha-[[[3-(4-hydroxyphenyl)-1-methylpropyl] amino]methyl] benzenemethanol	97825-25-7		$C_{18}H_{23}NO_3$	301.39	124	Fairly soluble

Table 14.6 Main properties of relevant antibiotics

Substance	IUPAC name	CAS number	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Sulfonamides						
Sulfacetamide	N-[(4-aminophenyl)sulfonyl]-acetamide	144-80-9	$C_8H_{10}N_2O_3S$	214.24	182	Slightly soluble
Sulfadiazine	4-amino-N-2-pyrimidinylsulfanilamide	68-35-9	$C_{10}H_{10}N_4O_2S$	250.28	252	Sparingly soluble in warm water
Sulfadoxine	4-amino-N-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide	2447-57-6	$C_{12}H_{14}N_4O_4S$	310.34	190	Very slightly soluble
Sulfadimethoxine	4-amino-N-(2,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide	122-11-2	$C_{12}H_{14}N_4O_4S$	310.33	201	Soluble in slight basic warm water
Sulfachlorpyridazine	4-amino-N-(6-chloro-3-pyridazinyl)benzenesulfonamide	80-32-0	$C_{10}H_9ClN_4O_2S$	284.74	190–191	Soluble
β-lactam antibiotics						
Amoxicillin	[2S-[2α,5α,6β(S*)]]-6-[[amino(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	26787-78-0	$C_{16}H_{19}N_3O_5S$	365.41	194	Slightly soluble
Penicillin G calcium salt	[2S-(2α,5α,6β)]-3,3-dimethyl-7-oxo-6[(phenylacetyl)amino]-4-thia-1-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid calcium salt	61-33-6	$(C_{16}H_{17}N_2O_4S)_2Ca$	706.84	–	Soluble
Penicillin V	3,3-dimethyl-7-oxo-6-[(phenoxyacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	87-08-1	$C_{16}H_{18}N_2O_5S$	350.38	120–128	Very slightly soluble in acid water
Tetracyclines						
Tetracycline	4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-	60-54-8	$C_{22}H_{24}N_2O_8$	444.43		

Table 14.6 (continued)

Substance	IUPAC name	CAS number	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
	methyl-1,11-dioxo-2-naphthacenecarboxamide					
Oxytetracycline	4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide	79-57-2	$C_{22}H_{24}N_2O_9$	460.44		
Chlortetracycline	7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide	57-62-5	$C_{22}H_{23}ClN_2O_8$	478.88		Slightly soluble
Aminoglycosides						
Dihydrostreptomycin	2-[(1*S*,2*R*,3*R*,4*S*,5*R*,6*R*)-5-(Diaminomethylideneamino)-2-[(2*R*,3*R*,4*R*,5*S*)-3-[(2*S*,3*S*,4*S*,5*R*,6*S*)-4,5-dihydroxy-6-(hydroxymethyl)-3-methylaminooxan-2-yl]oxy-4-hydroxy-4-(hydroxymethyl)- 5-methyloxolan-2-yl]oxy-3,4,6-trihydroxycyclohexyl]guanidine	128-46-1	$C_{21}H_{41}N_7O_{12}$	583.62		Soluble
Gentamycin	Various: gentamycin C_1; gentamycin C_2; gentamycin C_{1a} or D; gentamycin A	1403-66-3	–	–		Soluble
Streptomycin	5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)- 4-[4,5-dihydroxy-6-(hydroxymethyl)-3-methylamino-tetrahydropyran-2-yl] oxy-3-hydroxy-2-methyl-tetrahydrofuran-3-carbaldehyde	57-92-1	$C_{21}H_{39}N_7O_{12}$	581.58		Soluble
Streptomycin B		128-45-0	$C_{27}H_{49}N_7O_{17}$	743.72		Soluble

gram-positive and gram-negative bacteria, acting on specific targets in bacterial DNA synthesis (Croubels, Daeselaire, De Baere, De Backer, & Courtheyn, 2004), and have been used in human medicine for the treatment of systemic bacterial diseases even though they have been replaced by modern antibiotics but some like sulfamethazine (also known as sulfamidicine) are still used in animals due to low cost, easy administration, and high efficiency (Dixon, 2001).

β-Lactams

The chemical structure is based on the *β*-lactam ring. This group includes penicillins, *β*-lactamase inhibitors, and cephalosporins but also other subfamilies like cephamycines and clavulanic acid (see Table 14.6). They act on the growth of gram-positive bacteria by disrupting the development of bacterial cell wall. The *β*-lactams can also increase the feed efficiency and thus promote growth.

Tetracyclines

These are broad spectrum antibiotics with high activity against gram-positive and gram-negative bacteria, derived from certain *Streptomyces* spp., that act on bacterial protein synthesis. They can be used to treat respiratory disease in farm animals. At low doses can exert growth promotion effects in animals. Tetracycline, oxytetracycline, and chlortetracycline are some of the most well-known compounds in this group used in veterinary medicine (see Table 14.6).

Aminoglycosides

These antibiotics, which have a broad spectrum of activity, act against the synthesis of bacterial cell proteins in gram-negative bacteria. They are based on aminosugars linked by glycoside bridges to a central aglycone moiety. Streptomycin and dihydrostreptomycin belong to the streptomycin subgroup, while gentamicin and neomycin belong to the deoxystreptamine subgroup. They have different subclasses depending on the substituents to the deoxystreptamine moiety (i.e., neomycin A, B, or C) as shown in Table 14.6.

Macrolides

They were used to treat respiratory diseases, especially erythromycin. The structure is based on a macrocyclic lactone ring having carbohydrates attached. They are produced from certain *Streptomyces* strains. Macrolides act against gram-positive bacteria. Erythromycin is a good representative of this group. Tylosin, spiramycin, and lincomycin are also typical compounds belonging to this group which have been used for growth promotion (see Table 14.7).

Table 14.7 Main properties of relevant macrolides and quinolones

Substance	IUPAC name	CAS number	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Macrolides						
Tylosin	2-[12-[5-(4,5-dihydroxy-4,6-dimethyl-oxan-2-yl)oxy-4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl]oxy-2-ethyl-14-hydroxy-3-[(5-hydroxy-3,4-dimethoxy-6-methyl-oxan-2-yl)oxymethyl]-5,9,13-trimethyl-8,16-dioxo-1-oxacyclohexadeca-4,6-dien-11-yl]acetaldehyde	1401-69-0	$C_{46}H_{77}NO_{17}$	916.14	128–132	Slightly soluble
Erythromycin	6-(4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl)oxy-14-ethyl-7,12,13-trihydroxy-4-(5-hydroxy-4-methoxy-4,6-dimethyl-oxan-2-yl)oxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione	114-07-8	$C_{37}H_{67}NO_{13}$	733.92	135–140	Fairly soluble
Spiramycin	Complex: spiramycin I, II and III	8025-81-8	I: $C_{43}H_{74}N_2O_{14}$ II: $C_{45}H_{76}N_2O_{15}$ III: $C_{46}H_{78}N_2O_{15}$	I: 843.08 II: 885.12 III: 899.15	134–137	Slightly soluble
Quinolones						
Enrofloxacin	1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxilic acid	93106-60-6	$C_{19}H_{22}FN_3O_3$	359.40	219-223	Slightly soluble
Sarafloxacin	6-fluoro-1-(4-fluorophenyl)-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid	98105-99-8	$C_{20}H_{17}F_2N_3O_3$	385.36	256	Soluble
Danofloxacin	1-Cyclopropyl-6-fluoro-7-[(1*S*,4*S*)-3-methyl-3,6-diazabicyclo[2.2.1]heptan-6-yl]-4-oxoquinoline-3-carboxylic acid	112398-08-0	$C_{19}H_{20}FN_3O_3$	357.37	263	Soluble

Quinolones

They act against the bacterial DNA–gyrase with a broad antibacterial activity. Oxolinic acid, flumequine, and nalidixic acid are compounds of the first generation. They are synthesized from 3-quinolone carboxylic acid. The second generation compounds, which are more potent, are fluoroquinolones like sarafloxacin, enrofloxacin, and danofloxacin, which display fluorescence (see Table 14.7). These substances are poorly soluble in water at neutral pH but increase their solubility at basic pH.

Peptides

These are large and complex molecules which are obtained from bacteria and molds. Some of them are nisin, bacitracin, colistin, avoparcin, polymirxin, and virginiamycin (see Table 14.8). They can interact with the bacterial cell wall, resulting in cell membrane damage (Croubels et al., 2004). These antibiotics often have a mixture of several molecules (i.e., bacitracin A or F). Avoparcin was banned in the EU in 1997, while bacitracin and virginiamycin were banned in 1999 due to the risk of transmission of antibiotic resistance to bacteria (Verdon, 2009).

Amphenicols

These are broad spectrum antibiotics. Chloramphenicol, thiamphenicol, and fluorfenicol are the main representatives of this group. Chloramphenicol was banned in late 1980 s due to its toxic effects

Carbadox and Olaquindox

These are antibacterial synthetic quinoxaline compounds which have been used as growth promoters. Carbadox has shown mutagenic and carcinogenic effects in animals while olaquindox is strongly mutagenic (Croubels et al., 2004). Both antibiotics, shown in Table 14.8, are rapidly converted into quinoxaline-2-carboxylic acid (QCA) and methyl-3-quinoxaline-2-carboxylic acid (MQCA), respectively. These metabolites are mutagenic and carcinogenic (Verdon, 2009).

Nitrofurans

These are synthetic compounds with a broad spectrum of activity against bacteria. They are furazolidone, furaltadone, nitrofurazone, and nitrofurantoin (see Table 14.9). These substances are used against gastrointestinal infections in farm animals but were banned due to its genotoxic and mutagenic properties. They are rapidly metabolized in the organism (i.e., semicarbazide from nitrofurazone) making its detection more difficult.

Table 14.8 Main properties of relevant peptides, carbadox, and olaquindox

Substance	IUPAC name	CAS number	Formula	Molecular mass (g/mol)	Melting point (°C)	Solubility in water
Bacitracin A	5-(1-(4-(14-((1*H*-imidazol-5-yl)methyl)- 20-(2-amino-2-oxoethyl)-11-benzyl-8-*sec*- butyl-17-(carboxymethyl)-3,6,9,12,15,18,21-heptaoxo-1,4,7,10,13,16,19-heptaazacyclohenicosan- 2-yl)butylamino)-3-methyl-1-oxopentan-2-ylamino)- 4-(2-(2-(1-amino-2-methylbutyl)-4,5-dihydrothiazole- 4-carboxamido)-4-methylpentanamido)-5-oxopentanoic acid	1405-87-4	$C_{66}H_{103}N_{17}O_{16}S$	1422.70	221–225	Soluble
Nisin A	–	1414-45-5	$C_{143}H_{230}N_{42}O_{37}S_7$	3354.12	–	Soluble at slight acid pH
Colistin	*N*-(4-amino-1-(1-(4-amino-1-oxo-1-(3,12,23-tris(2-aminoethyl)- 20-(1-hydroxyethyl)-6,9-diisobutyl-2,5,8,11,14,19,22-heptaoxo-1,4,7,10,13,18-hexaazacyclotricosan-15-ylamino)butan-2-ylamino)- 3-hydroxybutan-2-ylamino)-1-oxobutan-2-yl)-*N*,5-dimethylheptanamide	1066-17-7	$C_{52}H_{98}N_{16}O_{13}$	2797.32	200–220	–
Carbadox	(2-quinoxalinylmethylene)hydrazine carboxylic acid methyl ester N,N'-dioxide; 3-(2-quinoxalinylmethylene)carbazic acid methyl ester N,N'-dioxide	6804-07-5	$C_{11}H_{10}N_4O_4$	262.22	239.5	Insoluble
Olaquindox	N-(2-hydroxyethyl)-3-methyl-2-quinoxalinecarboxamide 1,4-dioxide	23696-28-8	$C_{12}H_{13}N_3O_4$	263.25	207-213	Slightly soluble

Table 14.9 Main properties of relevant nitrofurans

Substance	IUPAC name	CAS number	Structure	Formula	Molecular mass (g/mol)	Solubility in water
Furaltadone	5-(4-morpholinylmethyl)-3-[[(5-nitro-2-furanyl)methylene]amino]-2-oxazolidinone	139-91-3		$C_{13}H_{16}N_4O_6$	324.29	Slightly soluble
Furazolidone	3-[[(5-nitro-2-furanyl)methylene]-amino]-2-oxazolidinone	67-45-8		$C_8H_7N_3O_5$	225.16	Very slightly soluble
Nitrofurantoin	1-[[(5-nitro-2-furanyl)methylene]amino]-2,4-imidazolidinedione	67-20-9		$C_8H_6N_4O_5$	238.16	Very slightly soluble
Nitrofurazone	2-[(5-nitro-2-furanyl)methylene]-hydrazinecarboxamide	59-87-0		$C_6H_6N_4O_4$	198.14	Very slightly soluble

Other Veterinary Drugs

Antihelmintic Agents

The faeces of animals may contain eggs or larvae from worm parasites (helminths) that can be ingested by other animals, specially cattle and sheep, with pasture. These drugs act on the metabolism of the parasite. There are several groups like benzimidazoles (thiabendazole, albendazole) that had widespread use, imidazothiazoles (tetramisole, levamisole), avermectins (ivermectin, doramectin), and anilides (oxyclozanide, rafoxanide, and nitroxynil).

Anticoccidials, Including Nitroimidazoles

Coccidia parasites are transmitted by faecal infection, especially in farms. Anticoccidials are used in poultry to prevent and control coccidiosis, a contagious infection caused by parasites that causes serious effects such as bloody diarrhoea and loss of egg production. There are several groups of compounds against coccidiosis like nitrofurans, carbanilides, 4-hydroxyquinolones, pyrimidines, and the ionophores. Ionophores are polyether antibiotics used against coccidia parasites in poultry. They include monensin, salinomycin, narasin, and lasalocid.

Nitroimidazoles are obtained synthetically with a structure based on a 5-nitroimidazole ring. Main compounds are dimetridazole, metronidazole, ronidazole, and ipronidazole. They are toxic for the bacteria when the 5-nitro group is reduced to free radicals by nitro reductase of the anaerobic bacteria (Verdon, 2009). These compounds are mutagenic, carcinogenic, and toxic toward eukaryotic cells, and thus, have been banned in the EU in the 1990 s for use in food-producing animals

Sedatives

These compounds are used to control the stress in farm animals but after several weeks they can also induce some growth promotion by redistribution of fat to muscle tissue. Some compounds are carazolol, chlorpromazine, azaperone, and xylazine.

Corticoids

Corticoids are hormones of the adrenal cortex. They are used as antiinflammatory agent for therapeutic purposes. Derivatives of prednisolone constitute the most important group of synthetic corticoids. Corticosteroids are involved in many physiological roles, specially in carbohydrate metabolism and in control of water balance. They may exert some growth promotion when used in combination with other hormones or β-agonists. Some used corticoids for

such purposes are dexamethasone, betamethasone, flumethasone, cortisone, desoxymethasone, and hydrocortisone.

Causes of Concern Due to the Presence of Residues in Meat

The presence of residues of veterinary drugs, growth promoting agents, or its metabolites in meat and other animal-derived foodstuffs is causing concern during the last decades to the sanitary authorities in different countries, especially in the European Union. Main causes of concern for these residues are based on their potential adverse toxic effects to consumers or the promotion of antibiotic resistance to microorganisms. In the case of meat products, they may also contain different types of residues having its origin in the meat used as raw material (Reig and Toldrá, 2007, 2008).

The European Food Safety Authority has recently issued an opinion about the contribution of residues in meat and meat products of substances with hormonal activity, specifically testosterone, trenbolone acetate, zeranol, and melengestrol acetate. A quantitative estimation of risk to consumers could not be established even though the individual epidemiological and toxicological data available and the reported evidence in the literature for an association between some forms of hormone-dependent cancers and red meat consumption (EFSA, 2007).

Diethylstilbestrol was already related to cancer in the 1940 s. It is genotoxic and mutagenic and has been related to the development of premature telarche and ovarian cysts in humans (Lone, 1997). Zeranol is a potent estrogen receptor agonist (Takemura et al., 2007), resembling its action to estradiol (Leffers, Naesby, Vendelbo, Skakkebaek, & Jorgensen, 2001). β-agonists are substances well known for its effects on consumers like gross tremors of the extremities, tachycardia, nausea, headaches, and dizziness. These effects were reported in Italy after consumption of lamb and bovine meat containing residues of clenbuterol (Barbosa et al., 2005).

Antibiotics have been extensively used in recent decades in order to improve feed conversion and reduce toxins formation, resulting in a promotion of animal growth productivity. But some of these antibiotics may have adverse effect on consumers. So, furazolidone, a major metabolite of nitrofuran, has shown mutagenic and carcinogenic properties (Guo, Chou, & Liau, 2003). Chloramphenicol may cause an irreversible type of bone marrow depression that might lead to aplastic anaemia (Mottier, Parisod, Gremaud, Guy, & Stadler, 2003). Allergy may be caused by enrofloxacin (Pecorelli, Bibi, Fioroni, & Galarini, 2004). Tumor production has been reported for sulfamethazine and also some toxic effect of sulphonamides on the thyroid gland (Pecorelli et al., 2004).

Anticoccidials, which are used in poultry to prevent and control coccidiosis, may lead to the presence of coccidiostat residues in poultry products (Hagren

Connolly, Elliott, Lovgren, & Tuomola, 2005). Its safety margin is narrow due to its toxic effects on humans like the specific dilatation of coronary artery (Peippo, Lovgren, & Tuomola, 2005).

However, at this moment the main concern on the use of antibiotics for growth promotion is the potential emergence of drug-resistant bacteria and disruption of the colonization barrier of the resident intestinal micro flora (Cerniglia & Kotarski, 2005). Intestinal flora is essential for human physiology, food digestion, and metabolism of nutrients (Chadwick, George, & Claxton, 1992; Vollard & Clasener, 1994), but the proportion of major bacterial species can experience large variations depending on the type of diet (Moore & Moore, 1995). Thus, the intestinal microflora may change in density and composition after continuous exposure to antibiotics residues present in foodstuffs like meat and meat products. Any species of the indigenous microflora may develop antimicrobial resistance as well as impair colonization resistance. For instance, concern emerged about the possible contribution of avoparcin use in farm animals to the emergence of glycopeptide resistance in enterococci. As a consequence of the use of such glycopeptide antibiotic as a growth promoter, vancomycin-resistant enterococci (VRE) were reported to be commonly found in the commensal flora of food animals, on meat from these animals and in the commensal flora of healthy humans (van den Bogaard, Bruinsma, & Stobberingh, 2000). Another potential indirect effect would be the increased susceptibility to infection by pathogens like *Salmonella* spp. and *Escherichia coli* (Cerniglia & Kotarski, 1998).

Control of Veterinary Drugs and Growth Promoters Residues in Meat

The presence of residues and its associated potential harmful effects on human health makes the control of veterinary drug residues an important issue for consumer protection.

The control of veterinary drug residues in the United States is under the National Residue Program (NRP) which is administered by the USDA Food Safety and Inspection Service (FSIS). There are two programmes, the domestic and the import residue sampling programmes. The FSIS domestic residue sampling programme is focused on preventing the occurrence of violative residues in food-producing animals. This programme provides several sampling plans to verify and ensure that slaughter establishments are fulfilling their responsibilities under the HACCP regulation, and according to the regulations of the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). The import residue testing program is mainly determining the operativeness and effectiveness of the residue control programme of an exporting country (Ellis, 2004). For both programmes, the type of sampling consists of monitoring plans and surveillance plans. The Part Number 556 under title 21 Food and Drugs of the Code of Federal Regulations gives the tolerances for residues of new animal drugs in foods (CFR, 2008).

The tolerances are based upon residues of drugs in edible products of food-producing animals treated with such drugs (Byrnes, 2005).

Some of these substances like estradiol, progesterone, and testosterone are allowed in the United States under strict application measures and acceptable withdrawal periods. The use of certain growth promoters is allowed in other countries like Canada, Mexico, Australia, and New Zealand. However, the use of growth promoters is officially banned since 1988 in the European Union (EC, 1988).

The use of veterinary drugs in food animal species is strictly regulated in the European Union and, in fact, only some of them can be permitted for specific therapeutic purposes under strict control and administration by a veterinarian (Van Peteguem & Daeselaire, 2004). In the European Union, the monitoring of residues of substances having hormonal or thyreostatic action as well as β-agonists is regulated through the Council Directive 96/23/EC (EC, 1996). Member States have set up national monitoring programmes and sampling procedures following this Directive which establishes the measures to monitor certain substances and residues in live animals and animal products. Some residues may remain in the edible parts of an animal after administration of a veterinary drug. Table 14.1 lists the main veterinary drugs and substances with anabolic effect as defined in such Directive. Group A includes unauthorized substances with anabolic effect, while group B includes veterinary drugs some of them having established maximum residue limits (MRL). The MRL is based on the type and amount of residual substance in the foodstuff that cannot constitute any risk for the consumers. MRL may differ from one international authority to another. The residues to monitor involves not only the active substance and its degradation products but also its metabolites that may remain in the foodstuffs (Bergweff & Schloesser, 2003; Bergweff, 2005).

Commission Decisions 93/256/EC (EC, 1993a) and 93/257/EC (EC, 1993b) gave the criteria that the analytical methodology should follow for the adequate screening, identification, and confirmation of these residues. Commission Decision 2002/657/EC (EC, 2002) implemented the Council Directive 96/23/EC (EC, 1996) and is in force since 1 September 2004. This Decision provides rules for the analytical methods to be used in testing of official samples and specific common criteria for the interpretation of analytical results of official control laboratories for such samples. According to this Decision, a minimum number of identification points are required for the correct identification of the substance. These points are achieved depending on the analytical technique used (i.e., four identification points are achieved when using mass spectrometry for the detection of substances in group A but only three for those in group B). Other requirements are the relative retention of the analyte that must correspond to that of the calibration solution at a tolerance of $\pm 0.5\%$ for GC and $\pm 2.5\%$ for LC. New concepts were also given in this Decision that allows the determination of the level of confidence in the routine analytical result. These are the decision limit (CCα), defined as the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant and the

detection capability (CCβ) which is defined as the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β. Thus, both limits are very useful even though its determination is rather complex.

In summary, there are numerous analytical techniques which are available for the control of the presence of veterinary drug residues, including growth promoting substances, in meats. New instrumentation provides an increased sensitivity. These methodologies have been recently reviewed and available elsewhere (Toldrá & Reig, 2006; Reig & Toldrá, 2009,a,b; Verdon, 2009).

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Chapter 15
Priority Environmental Chemical Contaminants in Meat

Gianfranco Brambilla, Annalaura Iamiceli, and Alessandro di Domenico

Introduction

Generally, foods of animal origin play an important role in determining the exposure of human beings to contaminants of both biological and chemical origins (Ropkins & Beck, 2002; Lievaart et al., 2005). A potentially large number of chemicals could be considered, several of them deserving a particular attention due to their occurrence (contaminations levels and frequencies) and intake scenarios reflecting the differences existing in the economical, environmental, social and ecological contexts in which the "from-farm-to-fork" activities related to meat production are carried out (FAO – Food and Agriculture Organization, 2008).

For the reasons reported above, the prioritization of contaminants of potential relevance to meat safety should be adequately framed within a risk analysis, a scientific process targeted on public health protection, aiming to estimate how much of a contaminant consumers in general and/or population-sensitive groups (such as children or elderly people) may be exposed to without (appreciable) risk. Health risk assessment is commonly divided into four steps providing answers to the following questions: (1) hazard identification – what can go wrong? (2) hazard characterization – what are the consequences? (3) exposure assessment – how can it happen? (4) risk estimation – what is the likelihood the adverse effect would happen (Gaylor et al., 1997; FAO, 1997)?

Prioritization of Chemical Contaminants in Meat

When talking about meat, we should consider for contaminants prioritization with respect to food safety the following factors: the chemical potential to bioaccumulate in food-producing animals; the relevance for humans of the

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intakes via food of animal origin; and the ability of some chemicals to cause severe toxicological effects as a consequence of long-term exposures (UNEP – United Nations Environmental Program).

Many of the toxic substances sharing the aforesaid features have been already framed within the Stockholm Convention (2001) and conventionally identified as persistent organic pollutants (POPs). By definition, POPs are "chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment" (the Stockholm Convention, 2001) .

The environmental persistence of POPs, generally correlated to their chemical stability that makes these substances highly resistant to biological and chemical degradation, represents the main factor relating such chemicals to long-time exposure. Bioaccumulation magnitude depends on several factors, one being the solubility of the substance in lipids (Bernes, 1998). This feature strengthens the tendency of POPs to be concentrated in the fatty tissue of a living organism and they are found in food-producing animals at higher concentrations than those present in the environment and/or feedingstuffs (Hoogenboom, 2004).

Potential adverse effects on the environment and human health caused by exposure to POPs are of considerable concerns for governments, non-governmental organizations and the scientific community.

The worldwide dimension and relevance of POPs in meat production is enhanced not only by their environmental persistence and the capacity of covering long distances away from the point of release but also, within the context of this paper, by the potential to reach Consumers of different countries, via the world trade of feeds and food of animal origin of unreliable quality.

Concerted international measures were adopted to efficiently control the environmental release of POPs: the Stockholm Convention opened for signatures in 2001 and entered into force in May 2004 (EU Council, 2006); it provides an international framework, based on the precautionary principle, that seeks to guarantee the elimination of POPs or the reduction of their production and use.

The substances actually under the Convention include eight individual organochlorine pesticides, hexachlorobenzene, polychlorobyphenyls (PCBs), polychlorodibenzodioxins (PCDDs) and polychlorodibenzofurans (PCDFs), all present in Annex A (substances to be eliminated); in Annex B, the substances whose production and use are restricted are reported, whereas Annex C comprises substances unintentionally produced whose releases have to be reduced and finally eliminated.

At present a second group of chemicals (candidate POPs) is under consideration for inclusion in the Convention on the basis of their risk profile prepared by the POP Review Committee; a third group (proposed POPs) has been proposed for risk evaluation to the Review Committee. In Table 15.1 the compounds belonging to the three different groups are listed; Table 15.2 provides hints on

Table 15.1 Priority, candidate and proposed-for-inclusion POPs within the Stockholm Convention

Priority POPs[a]		
Aldrin	Endrin	Polychlorobiphenyls (PCBs)
Chlordane	Heptachlor	Polychlorodibenzodioxins (PCDDs)
DDT (*p,p′*-DDT)	Hexachlorobenzene (HCB)	Polychlorodibenzofurans (PCDFs)
Dieldrin	Mirex	Toxaphene
Candidate POPs[b]		
Chlordecone	Lindane (*gamma*-HCH)	Perfluorooctane sulfonate (PFOS)
Hexabromobiphenyl	Pentabromodiphenyl ether	
Proposed for inclusion POPs[b]		
alpha-HCH	Octabromodiphenyl ether	Short-chained chlorinated paraffins
beta-HCH	Pentachlorobenzene	

[a] All compounds are targeted for "elimination", with the exception of DDT ("restricted use") and PCDDs and PCDFs ("unintentional production"). In most cases, production and/or use are subject to specific exemptions, likely reflecting local requirements.
[b] Updating of Annex A, B or C of the Convention by the POPs Review Committee. Third meeting of the POPs Review Committee (POPRC-3) 19–23 November 2007, Geneva, Switzerland.

their main physical–chemical and toxicological properties. The inclusion of organic chemicals in the frame of the Convention presumes that the above-mentioned science-based requirements (persistence, toxicity, bioaccumulation, long-range transport) should be met.

Factors Influencing the Exposure of Meat Animals to Chemical Contaminants

Meat production scenarios are driven by a variety of variable factors linked to each whose final result could enhance or reduce the risk of exposure and bioaccumulation of those contaminants of priority relevance in food-producing animals.

Schematically they can be summarized as follows: (a) the globalization of the markets of feeds and foods produced in countries that may be acknowledged for differences in environmental risk (Reardon & Barrett, 2002) (Fig. 15.1); (b) the proposition of feed materials innovative for origin and provenience (Brambilla & De Filippis, 2005; Sapkota, Lefferts, McKenzie, & Walker, 2007), to improve the meat nutritional quality (less fat with a modified composition in favour of (poly)unsaturated fatty acids with respect to cholesterol and saturated fats) (Givens, 2005), that could determine a lower dilution in the fat mass of lipophylic contaminants; (c) a cost increase of some agriculture practices that determine to reconsider the use of wastes (i.e. the turnover from more expensive chemically synthesized fertilizers to

Table 15.2 Synopsis of the relevant chemical, physical and toxicological features of consolidated, candidated and proposed POPs (WHO, 2006; Stow, 2005; IARC)

Compound	Structure	Molecular weight	$LogK_{OW}$	Half-life in soil (years)	Non-carcinogenic chronic toxicity	Guidance values	IARC group
Aldrin (CAS 309-00-2)		365	5.17–7.4	<1.6	Reproductive and developmental toxicity, neurotoxicity	WHO pTDI 0.1 μg/kg b.w. per day cumulative with Dieldrin ASTDR MRL – 0.03 μg/kg/day USEPA RfD – 0.03 μg/kg/day	3
Chlordane (CAS 57-74-9)	Cis Trans	410	4.58–5.57	4	Reproductive and developmental toxicity, neurotoxicity	WHO pTDI 0.1 μg/kg/b.w.	2B
pp′-DDT[a] (CAS 50-29-3)		355	6.19	15	Reproductive and developmental toxicity, neurotoxicity	WHO pTDI – 10 μg/kg/day USEPA RfD – 0.5 μg/kg/day Health Canada pTDI – 20 μg/kg/day	2B
Dieldrin (CAS 60-57-1)		381	3.69–6.2	3–4	Reproductive and developmental toxicity, neurotoxicity	WHO pTDI 0.1 μg/kg b.w. per day cumulative with Dieldrin ASTDR MRL – 0.03 μg/kg/day USEPA RfD – 0.03 μg/kg/ day	3

Table 15.2 (continued)

Compound	Structure	Molecular weight	$LogK_{OW}$	Half-life in soil (years)	Non-carcinogenic chronic toxicity	Guidance values	IARC group
PCDDs[b,c]		322–460	6.80–8.20	10–12[d]	Reproductive and developmental toxicity, immuno- and neurotoxicity	WHO TDI – 1–4 pg TEQ/kg/day (cumulative with PCDF and DL-PCBS) ASTDR MRL – 1 pg TEQ/kg/day	1
PCDFs[e,f]		306–444	6.53–8.7	—	Reproductive and developmental toxicity, immuno- and neurotoxicity	WHO TDI – 1–4 pg TEQ/kg/day (cumulative with PCDF and DL-PCBS) ASTDR MRL – 1 pg TEQ/kg/day	1
Endrin (CAS 72-20-8)		381	3.21–5.34	12	Reproductive and developmental toxicity, neurotoxicity	WHO pTDI – 0.0002 µg/kg/day ASTDR MRL – 0.3 µg/kg/day USEPA RfD – 0.3 µg/kg/day	3
Hexachlorobenzene (CAS 118-74-1)		285	3.93–6.42	2.7–5.7	Reproductive and developmental toxicity, neurotoxicity	WHO ADI – 0.17 µg/kg/day	2 B

Table 15.2 (continued)

Compound	Structure	Molecular weight	$LogK_{OW}$	Half-life in soil (years)	Non-carcinogenic chronic toxicity	Guidance values	IARC group
Heptachlor (CAS 76-44-8)		373	4.4–5.5	0.75–2	Hepatic, reproductive and developmental toxicity	WHO ADI – 0.5 μg/kg/day USEPA RfD – 0.5 μg/kg/day	2B
Mirex (CAS 2385-85-5)		546	5.28	10	Reproductive a nd developmental toxicity, immuno- and neurotoxicity	ASTDR MRL – 0.8 μg/kg/day USEPA RfD – 0.2 μg/kg/day Health Canada pTDI – 0.07 μg/kg/day	2B
PCBs[g]		189–499	4.3–8.26	> 6	Reproductive and developmental toxicity	ASTDR MRL – 0.02 μg/kg/day USEPA RfD – 0.02 μg/kg/day Health Canada pTDI – 1 μg/kg/day	2A
Toxaphene (CAS 8001-35-2)		414		0.3–12 years	Reproductive and developmental toxicity, immuno- and neurotoxicity	Health Canada pTDI – 0.2 μg/kg/day	2B

Table 15.2 (continued)

Compound	Structure	Molecular weight	$LogK_{OW}$	Half-life in soil (years)	Non-carcinogenic chronic toxicity	Guidance values	IARC group
Chlordecone (CAS 143-50-0)		490.6	4.50–5.41	1–2	Hepatic, reproductive and developmental toxicity	ASTDR MRL – 0.5 μg/kg/day	2B
Hexabromobiphenyl[h] (CAS 6355-01-8)		627.58	6.39	–	Hepatic, reproductive, immuno- and thyroid toxicity		2B
Lindane (*gamma*-hexachlorocyclohexane) (CAS 58-89-9)		290.83	3.8	>1	Reproductive and developmental toxicity, neurotoxicity	WHO tADI γ-HCH – 0–1 μg/kg/day	2B
PFOS[i] (CAS 2795-39-3)		538	Not measurable	–	Hepatic, reproductive and immuno (thymus) toxicity		
Penta-BDE[k] (commercial mixture)		485.8–564.7	5.9–7.0	–	Hepatic, reproductive and thyroid toxicity		

Table 15.2 (continued)

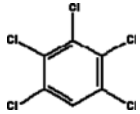
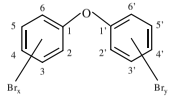
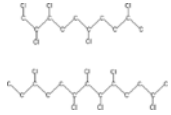
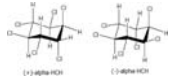
Compound	Structure	Molecular weight	$LogK_{OW}$	Half-life in soil (years)	Non-carcinogenic chronic toxicity	Guidance values	IARC group
Pentachloro-benzene (CAS 608-93-5)		250.32	4.8–5.18	194–345	Hepatic, nephric, hematological and developmental toxicity		2B
Octa-BDE[l] (commercial mixture) (CAS 32536-52-0)		801.38	6.29	–	Fetotoxicity, delayed fetal skeletal ossification, hepatic and thyroid toxicity		2B
SCCPs[m] (CAS 85535-84-8)		320–500	4.39–8.69	>365	Hepatic toxicity		2B
alpha-HCH[n] (CAS 319-84-6)		290.83	3.46–3.85	48–125	Neuro-, hepato-, immuno-toxicity	ASTDR MRL α-HCH – 8 μg/kg/day	2B

Table 15.2 (continued)

Compound	Structure	Molecular weight	$LogK_{OW}$	Half-life in soil (years)	Non-carcinogenic chronic toxicity	Guidance values	IARC group
beta-HCH[o] (CAS 319-85-7)		290.83	3.78–4.50	91–122	Hepatic reproductive toxicity		2B

[a] 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane.
[b] Polychlorinated dibenzo-*p*-dioxins.
[c] Data refer to the seven 2,3,7,8-chlorosubstituted toxic congeners only.
[d] Data refer to 2,3,7,8-T4CDD.
[e] Polychlorinated dibenzofurans.
[f] Data refer to the ten 2,3,7,8-chlorosubstituted toxic congeners only.
[g] Polychlorinated biphenyls.
[h] Only one isomeric structure is shown.
[i] Perfluorooctane sulfonate (the potassium salt is shown).
[k] Pentabromodiphenyl ether. The commercial mixture contains penta- through heptabromo-substituted homologues.
[l] Octabromodiphenyl ether. The commercial mixture contains penta- through decabromo-substituted homologues.
[m] Short-chained chlorinated paraffins.
[n] alpha-Hexachlorocyclohexane.
[o] beta-Hexachlorocyclohexane.
ASTDR, Agency for Toxic Substances and Disease Registry.
MRL, minimum risk levels for chronic exposure.
USEPA RfD, United States Environmental Protection Agency Reference doses.
WHO tADI, World Health Organization's temporarily acceptable daily intake.
WHO (TDI/pTDI), World Health Organization's (provisional) tolerable daily intake.
Health Canada (pTDI) provisional daily intakes.
IARC, International Agency of Research on Cancer; 1: The agent is carcinogenic to humans; 2A: The agent is probably carcinogenic to humans; 2B: The agent is possibly carcinogenic to humans; 3: The agent is not classifiable as to its carcinogenicity to humans; 4: The agent is probably not carcinogenic to humans.

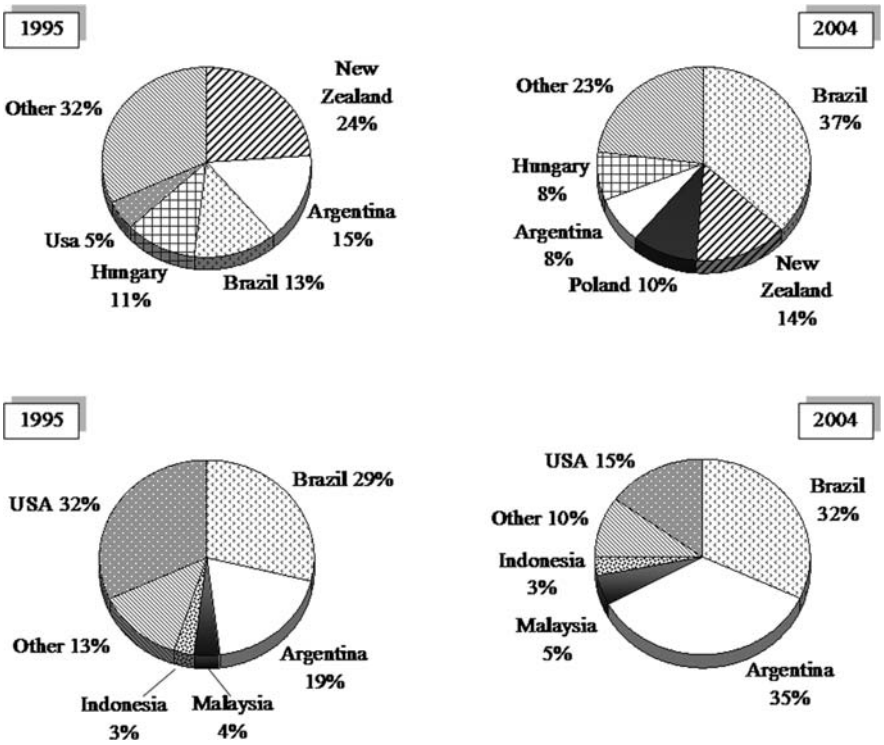


Fig. 15.1 Changes in meat (*above*) and feedingstuffs (*below*) imports from third countries to the European Union, 1 in 1994 and 2004 (Eurostat, 2006)

cheaper potentially contaminated sludges (Fiedler, Hutzinger, Welsch-Pausch, & Schmiedinger, 2000; Schoof & Houkal, 2005); (d) new trends in animal managements, towards less intensive farming systems, and an improved welfare, that could lead to an increased exposure to environmental contaminants (i.e. through forages and soils) with respect to that coming from feedingstuffs placed on the market (Schierea, Ibrahim, & van Keulenc, 2002) (FAO, 2000); (e) climate changes and new evaluations of the risk/benefit ratio in the use of pesticides, such as dichlorodiphenyltrichloroethane (DDT) to prevent arthropod-borne transmissible diseases both to animals and to humans (FAO, 2008; WHO, 2007); (f) not sufficiently implemented protective farming practices with respect to possible backyard emissions due to the improper disposal of agriculture wastes (Codex Alimentarius, 2006; United States Environmental Protection Agency – US EPA, 2008). In Tables 15.3 and 15.4 an inventory of the ports of entry for "dioxins" and other POPs in the food chain are, respectively, reported (Brambilla, Iamiceli, Ferri, & di Domenico, 2008).

Table 15.3 Inventory of potential sources of exposure to PCDDs, PCDFs and PCBs in farmed animals

Item	Origin of contamination
	Feedingstuffs (in market)
1. Anticaking agents (clay)	• Waste from high-temperature furnaces
2. Mineral components	• Ashes from rotary kilns
3. Zinc and copper oxide	• Hydrolysis of bones and shells with hydrochloric acid used in PVC production
4. Calcium phosphate	
5. Rendered oils for animal nutrition	• Contamination with heat-stressed or aged PCB-containing dielectric fluids • Contamination from exhausted gasoline or combustion oil residues in barrels
6. Fish oils and meals	• Contaminated aquatic webs
	Forages (in farm)
1. Drying of feed material	• Open flame devices
2. Crop harvesting	• Crop exposure to contaminated soil
3. Atmospheric "fall-out"	• Improper disposal (burning) of agricultural waste
	Grasslands and water
1. Grazing fields	• Use of sewage sludge as fertilizers • Use of incineration ashes as mineral fertilizers • Vicinity to chloro-pesticide plants/stores • "Fall-out" from local regular emissions (metal smelters, plastic material manufacturers, etc.) • Destruction of animal carcasses • Improper disposal (burying) of agricultural and veterinary wastes
2. Water	• Vicinity to paper mills, to chlorinated pesticide factories; releases from pesticide stores
	Farm work and indoor areas
1. Stable	• Leakage from old electrical apparatuses • Timber treated with antifouling agents (copper chromium arsenate (CCA), pentachlorophenol (PCP), etc.)
2. Bedding	• CCA- and PCP-contaminated shavings and dust
3. Anti-slippery floors	• Recycled PVC, rubber and plastic materials

From Residues Monitoring Plans to Intake Assessment

Although many POPs are already strictly regulated or are no longer in production, as the food of animal origin still represents the main source of exposure, the measurement of POPs in food and, in particular, in products of animal origin is particularly relevant for the protection of human health and for the consumers' perception about food safety. Appropriate monitoring plans and maximum residue limits (MRLs) for some POPs (organochlorine pesticides) in a variety of food commodities were established by European Union (EU) and

Table 15.4 Potential sources of exposure to candidate POPs in meat animals (UNEP, 2002)

Compound	Use	Exposure source
1. Chlordecone	Insecticide, fungicide and degradation product of insecticide mirex	• Contamination of crops and seeds
2. Dicofol	Acaricide structurally similar to DDT, used on grapes, beans, cotton, pumpkin, ornamental plants, melon and watermelon	• Contamination of forages • Fish oils
3. Endosulfan	Insecticide and acaricide (against mites) used in horticulture, crop industry and cotton	• Forages (feed) contamination
4. Hexabromobiphenyl	Flame retardant in thermoplastics for industrial and electrical products	• Top soil treated with sewage sludge • Fish oils • Contact materials
5. Hexachlorocyclo-hexanes (*alpha*-, *beta*-, *gamma*-HCH)	• Insecticide for treatment of seeds, lice and scabies • Used on soils intended for sugarbeet cultivation	• Feed contamination • Soil and water contamination • Fish oils and meals
6. Pentachlorobenzene	Obsolete pesticide, flame retardant and intermediate to make fungicides	• Closeness to product stock piles • Fish oils and meals
7. Perfluoro-organic compounds (PFCs, PFOS, PFOA, etc.)	Stain resistance treatments for fabrics/paper, coatings for metal surfaces including non-stick cookware and electronics components, fire fighting foams	• Water • Topsoil treated with sewage sludge • Fish meals • Litter from recycled paper
8. Polybromodiphenyl ethers (PBDEs)	Flame retardants in plastics for TV sets and computers, in carpets, car interiors and polyurethane foams for furniture and bedding	• Contact materials (rubbers, carpets) • Topsoil treated with sewage sludge • Fish oils (meals)
9. Short-chained chlorinated paraffins (SCCPs)	High-temperature lubricants, plasticizers, flame retardants and additives in adhesives, paints, rubbers and sealants	• Fish oils • Topsoil treated with sewage sludge
10. Polychloronaphtha-lenes (PCNs)	Cable insulation, wood preservative, engine oil additive, capacitor fluids, dye intermediate and flame retardants	• Contact material (bedding) • Topsoil treated with sewage sludge

non-EU countries, thus making mandatory the development of sensitive methods to analyse these pollutants in food, along with the establishment of correct sampling procedures and sample pre-treatments (Food and Agriculture Organization/World Health Organization – FAO/WHO, 2001; EU Regulation, 2005).

More recently, international and national bodies have focused their attention on alimentary exposure (intake) assessment (WHO, 2000; 2002), trying to verify to what extent the regulatory actions are effective in keeping consumers and sensitive population groups from unacceptable levels of exposures. Schematically, such an assessment may be carried out following two different approaches. The first is a so-called indirect approach or dietary modelling: dietary modelling is the process of combining country-based food consumption data (European Food Safety Authority – EFSA, 2008) and chemical concentration data in foods (i.e. those data coming from residue monitoring plans) to estimate the intake of the selected contaminants. Intake estimates are then compared to reference health standards for life-long exposures, usually expressed as "tolerable daily, weekly or monthly intakes" (TDI, TWI and TMI) to estimate the risk to population health (WHO, 1999). These estimates may be carried out on a deterministic (average or worst-case exposures) or a (semi-) probabilistic basis, taking into account the distribution curves of the data and the relative percentiles (Kroes et al., 2002). However, they are affected to different extents by uncertainties that suggest a prudent use of the exposure outcomes.

Uncertainties may arise from both the quality and the representativeness of the data, such as

- sampling strategy focused on meat batch and consignment and not on the food really eaten by consumers;
- data not consistent enough to describe the distribution of contamination for each category of food considered in the consumption database;
- food of animal origin with different fat content grouped in the same class of food item (i.e. meat and meat products);
- analytical methods basically focused on contamination values only around the MRL range, sufficient to give a compliance/non-compliance evaluation, but not validated for determination over the entire range of possible contamination levels.

To overcome the aforesaid biases in dietary modelling, some countries have planned specific studies (the so-called total diet studies (TDS) or "market basket studies") that involve the purchasing of food samples at retail level, according to their representativeness in the food diet of a selected population and their preparation according to national household procedures. The foods so prepared are then aggregated for macro categories (fish and fishery products, milk and dairy products, egg and egg-based products, meat) and the resulting composite samples analysed for the contaminants of interest (WHO, 2002).

The second (direct) approach is represented by the "duplicate diet" (DD) methodology (Thomas et al., 1997); selected population groups as representative of country food habits are asked to duplicate their meals within a fixed time frame (e.g. 1 week), meals that will be analysed. Such direct approach provides at the same time accurate information on both dietary habits and contaminant intakes Moreover, it takes into account possible factors that may influence the contaminant content, such as meat cooking process (e.g. frying, boiling, roasting,

steaming) (Noël, Leblanc, & Guérin, 2003). However, the necessity of an active check of consumers may affect the representativeness of the outcomes due to the rather limited number of observations usually carried out in such studies.

In the following sections, the relevant information about chemistry, occurrence, exposure and analysis of the main categories of priority chemical contaminants in meat will be provided.

Organochlorine Pesticides

The family of organochlorine pesticides groups a wide range of organic chemicals containing chlorine atoms, used in agriculture and public health to effectively control pest. Although most of them were during the 1970 s and 1980 s, they are still found in the environment (Rhind, 2002; Konstantinou, Hela, & Albanis, 2006) and in biological matrices (Torres et al., 2006; Meeker, Altshul, & Hauser, 2007). Due to their chemical–physical properties as POPs, food is considered to represent a long-term source of exposure. Current EU MRLs established for the organochlorine pesticides of interest in animal products are set between 0.02 and 1 mg/kg fat. An inventory of the regulatory limits in different countries according to the animal species is reported in Table 15.5. The official occurrence data in EU meat are available in the binary form "compliant/non-compliant". However, in 2004, of 436 samples targeted on different types of meat, a few non-compliant outcomes were found in cattle for gamma-hexachlorocyclohexane (HCH) (lindane) residues (three results), pigs (one for beta-HCH and one for DDT, as the sum of its isomers and related compounds) and sheep (two for beta-HCH) (EU Commission, 2004). At international level, MRLs in meat and meat products recommended by Food and Agriculture Organization (FAO) jointly with World Health Organization (WHO) vary from 0.05 to 3 mg/kg fat. In the United States, legislation was enacted in 1996 with the Food Quality Protection Act, including stricter safety standards, especially for infants and children, and a complete reassessment of all existing pesticide tolerances. For the pesticides of our concern, US residue limits are established between 0.1 and 7 mg/kg fat. The US Pesticide Monitoring Program does not focus on meat, but uses primarily cow milk and eggs as the more relevant animal food source of exposure and biomarkers.

A Swedish market basket survey (Darnerud et al., 2006) supports the evidence that meat products do not represent a relevant source for organochlorine pesticide intake; for DDT (sum of p,p′-DDE, p,p0-DDD, p,p′-DDT and ṕ-DDT), meat contribution to intake is on average 83 ng on a total dietary intake of 524 ng (15% of contribution), whereas for the three HCH and for the four chlordane congeners the ratio (in nanograms) is 9.5/81 and 6.2/115, corresponding to 12 and 5% contributions, respectively.

Multi-residue procedures and highly sensitive methods are currently a requirement in organochlorine pesticide analysis in products of animal origin. Hercegová,

Table 15.5 Regulatory limits (MRLs) in μg/g fat for residues of POP pesticides in meat in EU and several non-EU countries

POP	Australia	EU[a]			FAO[b]	Japan	Korea	United Kingdom	United States
		Meat	Offals	Fat					
Aldrin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3
Chlordane	0.2	0.05	0.05	0.05	0.05	0.08	0.5	0.05	0.3
DDT	5	1	1	1	5	1	5	1	5
Dicofol	–	0.05–0.5	0.05	0.05	3	0.3–0.08	–	0.05–0.5	–
Dieldrin	0.2	0.2	0.2	0.2	0.2	0.2	–	0.2	0.3
Endrin	–	0.05	0.05	0.05	–	0.05	0.1	0.05	0.3
HCB	1	0.2	0.2	0.2	–	0.2	–	0.2	0.5
HCH	–	0.3	0.3	0.3	–	–	–	0.1–0.2	–
Heptachlor	0.2	0.2	0.2	0.2	0.2	0.2	–	0.2	0.2
Lindane[c]	2	0.02	0.02	0.02	0.1	0.02	2	0.02	4–7
Mirex	–	–	–	–	–	–	–	–	0.1
Toxaphene	–	0.05	0.05	0.05	–	–	–	0.05	–

[a] Legislative reference (harmonization in progress within the framework of Regulation 2005/396/EC). For aldrin, chlordane, DDT, dieldrin, endrin, HCB, HCH, heptachlor: Directive 1993/57/EEC. For dicofol: Directives 1993/57/EEC and 2000/24/EC. For lindane: Directive 2002/66/EC. For toxaphene: Directive 2004/61/EC.
[b] Together with WHO.
[c] *gamma*-HCH.

Dömötörová, and Matisová (2007) have recently reviewed available methods and drawn the following decalogue: (a) possibility to determine a number of pesticides as high as possible in a single analysis; (b) high recoveries; (c) high selectivity obtained by means of an effective removal of potential interferences from the sample; (d) high sensitivity; (e) high precision; (f) good ruggedness; (g) low cost; (h) high speed; (i) use of less harmful solvents and in low amounts.

Multi-residue methods developed for organochlorine pesticides follow the general scheme shown in Fig. 15.2. After a sample pre-treatment

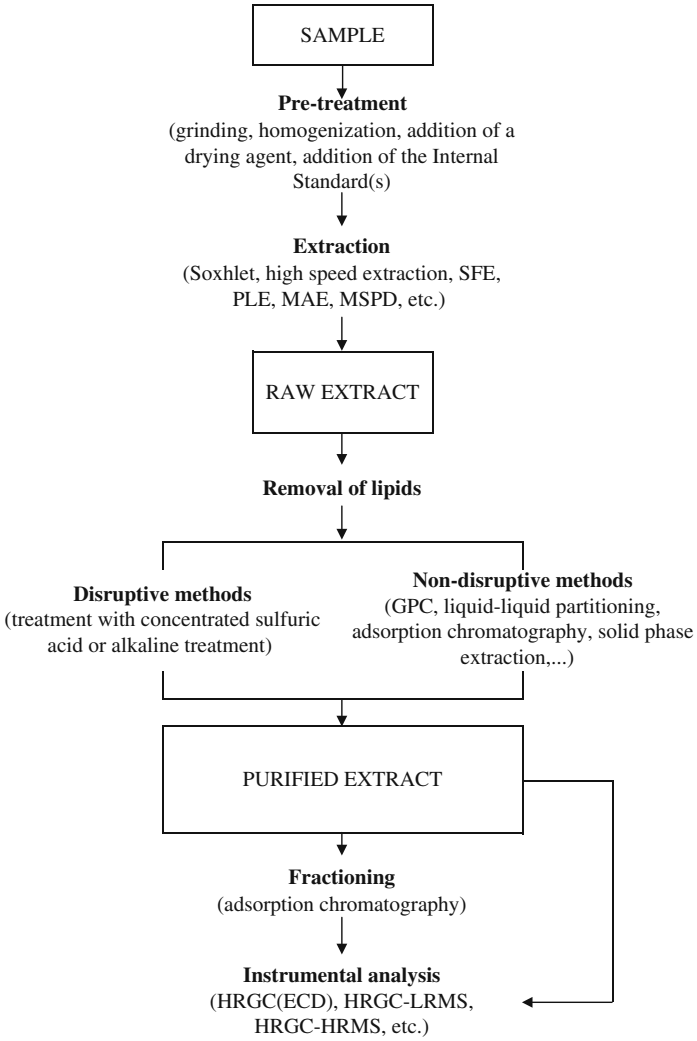


Fig. 15.2 General flow chart for the analysis of organochlorine pesticides, PCDDs, PCDFs, PCBs and PBDEs

(homogenization and drying), the analytes and fat are co-extracted. Lipids are separated from analytes using different non-destructive procedures, such as liquid–liquid partitioning and gel permeation chromatography (GPC). Adsorbent phases Florisil® (US Silica Company, Berkeley Springs), alumina or silica gel are then used for the clean-up step. Instrumental determination is performed by high-resolution gas chromatography (HRGC) coupled with electron capture detection (ECD) or with mass spectrometry (MS). The principal procedures currently used for the analysis of organochlorine pesticides in meat samples were reviewed by the Codex Committee on Pesticide Residues (Codex, 2003) and included in the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC International, 2005) and in the Pesticide Analytical Manual of the Food and Drug Administration (US FDA, 1994).

Non-dioxin-Like Polychlorobiphenyls (NDL-PCBs)

Polychlorinated biphenyls (PCBs) are a family of 209 chlorinated compounds, no longer produced and used in Europe, United States and Canada, but still commercially available under various trade names in other parts of the world. PCBs are substantially insoluble in water, very lipophilic and highly persistent (Table 15.2). For their chemical–physical stability and dielectric properties, they were used worldwide as dielectric fluids in transformer and capacitor oils, as hydraulic and heat exchange fluids and as lubricating and cutting oils. Non-dioxin-like (NDL)-PCBs have a different toxicological profile from PCDD, PCDF and dioxin-like (DL)-PCBs.

In experimental animal studies carried out with individual NDL-PCB congeners (PCB 28, 128 and 153 at a dose range of 30–40 μg/kg-bw per day) showed liver and thyroid toxicity to be the most sensitive toxicological end-points. However, because in field conditions a contamination with both NDL-PCB and DL-PCBs could occur, possible confounding effects can be determined by the simultaneous presence of DL and NDL congeners, owing to toxicological end-points being almost the same. In a recent opinion EFSA (2005) concluded that no health-based guidance value for humans could be established for NDL-PCB; however, regulatory levels as indicators of an overall good farming practices are suggested.

NDL-PCBs bioaccumulate in meat, liver and particularly in fat tissues of farmed animals and have been included in the EU national residue monitoring plans in food of animal origin since 1990. Data usually refer to the following six (Σ_6) NDL-PCB congeners (PCB 28, 52, 101, 138, 153, 180) as indicator or marker PCBs. The six individual congeners were not selected from a toxicological point of view, but considered to be representative for the different PCB patterns in various sample types. The Σ_6 NDL-PCB represents about 50% of total NDL-PCB in food. In some countries, the dioxin-like PCB 118 has been added to the former six, to form a group of seven (Σ_7) indicator PCBs. Lacking

toxicology and health-based guidance values, the EU Commission may set a MRL for the Σ_6 (NDL-PCBs) in meat products, considering its occurrence recorded from national residue control plans. A 99th percentiles around 48 and 28 ng/g fat have been recently reported for ruminant and pig meats, respectively (European Commission, DG Sanco working document, January 2008).

More than 90% of the NDL-PCB exposure in the general population is via food. Average dietary daily intakes of total NDL-PCB were estimated to be in the range of 10–45 ng/kg b.w. per day, excluding breastfed infants; children had exposure levels 2.5-fold higher than adults due to a larger intake of milk and dairy products. Moreover, some European (sub)-populations and other specific groups consuming fish-based diets may be exposed to twofold higher intakes than the average population. This determines the efforts to lower the levels of NDL-PCB in foods (EFSA, 2005).

An inventory of intake estimates of NDL-PCBs was carried out by EFSA (2005) for three different European countries, by considering the average occurrence in food items, their average fat contents and the average food consumption (Table 15.6). In the Netherlands (Baars et al., 2004), the estimated median life-long-averaged intake of the Σ_6 NDL-PCBs was 5.6 ng/kg b.w. per day, with a meat product contribution of 27%. These data indicated, however, a diminishing trend in the concentration of the contaminants in selected food items with respect to previous national studies. The same diminishing trend was reported by Fattore, Fanelli, Dellatte, Turrini, and di Domenico, (2008) for the Σ_6 NDL-PCBs dietary intake in the Italian population: the mean exposure resulted in 10.9 ng/kg b.w. in adults (13–94 years old). Fish and fishery products and milk and dairy products were the major contributors to the total dietary intake, with meat contributing at 10%.

As reported above, most analytical studies on NDL-PCBs are limited to the determination of a small number of congeners (PCBs 28, 52, 101, 138, 153 and 180) as indicators of the presence of all the chemical class. The analytical procedures for NDL-PCBs have recently been reviewed by Ahmed (2001), for PCB analysis in food. With respect to the analytical determination of "dioxins" (PCDDs and PCDFs) and DL-PCBs, differences may be observed in the chromatographic and detection systems utilized (Fig. 15.2). The general scheme consists on an organic solvent extraction; then lipids are removed by gel permeation chromatography (GPC) or treatment with sulfuric acid and co-extracted substances are eliminated by adsorption chromatography. A comprehensive review on developments in the high-resolution gas chromatography (HRGC) of PCBs is given by Cochran and Frame (1999), who evaluated a variety of stationary phases commonly used for PCB analysis. The 5%-phenyl type column has substantially become the standard for PCB analysis. The final determination is performed by HRGC-ECD (electronic capture detector) or, preferably, by HRGC-LRMS (low-resolution mass spectrometry). The internal standard (IS) technique is generally adopted in accord with the US EPA Methods 1668 (US EPA, 1999).

Table 15.6 Estimates of the dietary exposures for the average consumer to NDL-PCBs of three EU countries (EFSA, 2005)

Food groups	Sum PCBs (mean) ng/g	Consumption Italy	Consumption France	Consumption Sweden	Exposure Italy	Exposure France	Exposure Sweden
Cereals and cereals products	0.0213	270	218	292	6	5	6
Fruits and vegetables[a]	0.0495	498	313	387	25	15	19
Eggs[b]	0.73	18	17	15	13	12	11
Fats and oils[c]	5.05	38	18	24	192	91	121
Meat and meat products[d]	1.52	134	117	143	204	178	218
Offals[e]	0.74	3	3	7	2	2	5
Fish and fish products	12.50	43	32	35	538	400	438
Milk	0.17	124	106	343	21	18	59
Cheese and dairy[f]	0.98	87	100	45	86	98	44
Total (ng/day)					1086	821	921
Total (ng/kg b.w. per day					18.1	13.7	15.4

[a] Including potatoes.
[b] Assuming 11.1 g fat for 100 g product.
[c] An average value for vegetal oil was used.
[d] Assuming 12 g fat for 100 g meat.
[e] Assuming 10.9 g fat for 100 g liver.
[f] Assuming 1.6 fat for 100 ml milk.

Polychlorodibenzodioxins, Polychlorodibenzofurans and Dioxin-Like PCB (PCDDs, PCDFs and DL-PCBs)

PCDDs and PCDFs (altogether also commonly known as "dioxins") are not produced intentionally; in fact their formation and release into the environment occur primarily in thermal or combustion processes or as unwanted by-products of industrial processes involving chlorine. They form two groups of tricyclic aromatic compounds containing between one and eight chlorine atoms, thus resulting in 210 congeners (75 PCDDs and 135 PCDFs), different in the number and/or position of chlorine atoms; only the 17 congeners with chlorines at positions 2, 3, 7 and 8 are of toxicological interest. "Toxicity equivalency factors" (TEFs) have been proposed by different regulatory bodies (for instance, US EPA, NATO, WHO) since 1970 and applied to evaluate the cumulative effect of the toxicity on mammals or environmental species. In 1997 the World Health Organization (WHO) adopted the (WHO-TEF) based approach also for food and feedingstuffs to each of the 17 congeners: the highest TEF of 1 was assigned to 2,3,7,8-T_4CDD (as the most toxic congener) (Van den Berg et al., 1998): an update of the aforesaid WHO-TEFs was carried out in 2005 (van den Berg et al., 2006).

Due to the shared mode of action, WHO-TEFs have also been assigned to 11 PCB congeners, named as dioxin-like PCBs. In Table 15.7, the former consensus-based 1997 and the new adopted 2005 TEFs for all the PCDD, PCDF and PCB congeners with a dioxin-like activity are reported.

Humans are exposed to PCDDs, PCDFs and DL-PCBs mainly through the diet. The contribution of foods of animal origin (i.e. meat and fish and their products) may be higher than 90% of the total exposure to the aforesaid contaminants (Fattore, Fanelli, Turrini, & di Domenico, 2006; Tard, Gallotti, Leblanc, & Volatier, 2007). In order to reduce human exposure and protect consumer health, the EU has progressively issued regulatory measures setting maximum levels (MLs) and action levels (ALs) for PCDDs, PCDFs and DL-PCBs in food (Table 15.8). For example, a ML of 3.0 pgWHO-TE/g fat was established for PCDDs and PCDFs in bovine and sheep meat corresponding to a ML of 4.5 pgWHO-TE/g fat when DL-PCBs are considered (EU Regulation 1881, 2006); in pork meat, the corresponding ML values are 1.0 and 1.5 pgWHO-TE/g fat. When contaminant concentrations are greater than ALs but not MLs, the meat is not withdrawn from the market, but it is mandatory to trace back the source of exposure(s) that may have determined the unwanted contamination level higher than the average, i.e. through a feed.

Many data are presently available for their occurrence in carcasses from pigs and beefs. A former US EPA, United States Department of Agriculture (USDA) and Food and Drug Administration (FDA) survey carried out in 1994–1996 on 56 carcasses of pigs and 51 carcasses of steers and heifers found a mean 1.44 (median, 1.19) pgWHO-TE/g fat for PCDDs and PCDFs, and a mean 1.47 (median, 1.22) pgWHO-TE/g fat for PCDDs, PCDFs and DL-PCBs

Table 15.7 Comparison of the 1998 and 2005 TEFs for PCDDs, PCDFs and DL-PCBs; values in italics indicate a change in TEF value (Van den Berg et al., 2005)

Compound	1998 WHO-TEFs	2005 WHO-TEFs
Chlorinated dibenzodioxins		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01
OCDD	0.0001	*0.0003*
Chlorinated dibenzofurans		
2,3,7,8-TCDF	0.1	0.1
1,2,3,7,8-PeCDF	0.05	*0.03*
2,3,4,7,8-PeCDF	0.5	*0.3*
1,2,3,4,7,8-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01
1,2,3,6,7,8,9-HpCDF	0.01	0.01
OCDF	0.0001	*0.0003*
Non-*ortho*-substituted PCBs		
PCB 77	0.0001	0.0001
PCB 81	0.0001	*0.0003*
PCB 126	0.1	0.1
PCB 169	0.01	*0.03*
Mono-*ortho*-substituted PCBs		
PCB 105	0.0001	*0.00003*
PCB 114	0.0005	*0.00003*
PCB 118	0.0001	*0.00003*
PCB 123	0.0001	*0.00003*
PCB 156	0.0005	*0.00003*
PCB 157	0.0005	*0.00003*
PCB 167	0.00001	*0.00003*
PCB 189	0.0001	*0.00003*

together. Later data, referred to the 2002–2003 period, indicated a mean 0.24 (median, 0.15) pgWHO-TE/g fat for PCDDs and PCDFs in 136 pig carcasses and a mean 0.28 (median, 0.18) pgWHO-TE/g fat when DL PCB contributions are included. The mean level of PCDDs and PCDFs in heifer and steer carcasses (N = 139) was 0.79 (median, 0.73) pgWHO-TE/g lb, whereas the sum of PCDDs, PCDFs and DL-PCBs was found to be 0.93 (median, 0.56) pgWHO-TE/g fat (US FDA, 2006). An USDA market basket study (Huwe & Larsen, 2005) reported a PCDD and PCDF mean contaminations of 0.64, 1.54 and 0.37 pgWHO-TE/g fat for beef, hamburgers and pig meat, respectively; the

Table 15.8 MLs and ALs for meat and meat products according to Regulation 1881/2006/EC and Recommendation 2006/88/EC. Levels concern PCDDs plus PCDFs (I), DL-PCBs (II) or their sum (I + II), to be compared with upper bound analytical outcomes

Food or feed item	ML(I)	ML(I + II)	AL(I)	AL(II)
EU acceptance levels for meat and meat products[a]				
Ruminants (bovine, ovine)	3.0	4.5	1.5	1.0
Poultry and farmed game	2.0	4.0	1.5	1.5
Pork	1.0	1.5	0.6	0.5
Liver and liver products	6.0	12.0	4.0	4.0

[a] Values in pgWHO-TE/g fat (fat, >1%). The same levels apply to the fats derived from ruminants, poultry and pork.

corresponding DL-PCB mean concentrations were 0.11, 0.15 and 0.05 pgWHO-TE/g fat, respectively.

In 2000, the EU Scientific Committee on Food made an inventory on 138 meat products within the EU. In ruminants, the mean concentration of PCDDs and PCDFs was 0.74 pgWHO-TE/g fat and that of DL-PCBs 0.72 pgWHO-TE/g fat. In pigs, PCDDs and PCDFs were found at a mean 0.85 pgWHO-TE/g fat, whereas DL-PCB concentration was 0.40 pgWHO-TE/g fat (EC/EU SCF, 2000). New data coming from implementing EU national residue plans followed in 2004: the mean concentrations in ruminants for PCDDs and PCDFs alone, or together with DL-PCBs, were 0.46 and 0.80 pgWHO-TE/g fat, respectively. The corresponding values of 0.21 and 0.23 pgWHO-TE/g fat were reported for pigs (Gallani & Boix, 2004). Compared with the older data, the most recent monitoring results show a decrease of PCDD and PCDF levels, in line with the observed general trend (US EPA, 2006).

According to results of a national PCDD, PCDF and DL-PCB monitoring program, Australian Government (2005) reported total mean contaminations of 0.845, 0.575 and 0.803 pgWHO-TE/g fat in beef, pigs and lamb, respectively.

For the exposure assessment, guidance values as TDI, TWI and TMI have been set by different international bodies (Table 15.9). In most countries, the estimated mean intakes in adults seem close to such values, thus indicating a not

Table 15.9 Guidelines[a] for total human exposure to PCDDs, PCDFs and DL-PCBs. Values expressed in pgWHO-TE/kg body weight

Organization or country	TDI[b]	TWI[b]	TMI[b]
EU (2001)	*2*	**14**	*60*
WHO (1998)	**1–4**	*7–28*	*30–120*
JEFCA (2001)	*2.3*	*16.3*	**70**
NL (2000)	**1**	*7*	*30*
Japan (2000)	**4**	*28*	*120*
Australia (2002)	*2.3*	*16.3*	**70**

[a] Original guidelines in bold; values extrapolated by these authors in italics.
[b] TDI, TWI, TMI: tolerable daily, weekly or monthly intake.

negligible part of population may be over-exposed. However, a diminishing trend is noted with respect to previous studies, as already observed for NDL-PCBs. Among food items, meat consumption represents the third contribution in order of relevance to intake, after fish and fishery products and milk and dairy products, with some possible relevant differences according to the country or local food habits (Table 15.10).

The necessity to evaluate the cumulative presence of PCDDs, PCDFs and DL-PCBs along with their possible low contamination levels (in the order of pg/g WHO-TE) makes the analytical approach rather complex, time consuming and rather expensive. Reference methods have been elaborated by the US Environmental Protection Agency for the determination of the PCDD and PCDF toxic congeners (US EPA, 1994) and for DL-PCB congeners (US EPA, 1999) by HRGC-HRMS. The basic requirements for the EU official analytical methods to determine PCDD, PCDF and DL-PCB levels in foodstuffs are reported in Regulation 1883/2006 EC (2006). A critical review of the various methods used to analyse DL-PCBs is given by Iamiceli, Fochi, Brambilla, and di Domenico (2008). Many analytical methods follow the following

Table 15.10 Contribution of meat consumption to the mean total intake of PCDDs, PCDFs and DL-PCBs in different studies

Country (town)	Year	Congeners	Estimated intake	Average meat contribution (%)	References
Japan (Tokyo)	2004	PCDDs, PCDFs and DL-PCBs	1.55 pgWHO-TE/kg b.w. per day	11	Sasamoto et al. (2006)
Finland	2004	PCDDs, PCDFs and DL-PCBs	115 pg 1.5 pgWHO-TE/kg b.w.	5***	Kiviranta et al. (2004)
Australia	2004	PCDDs, PCDFs and DL-PCBs	3.7–15.6 pgWHO-TE/kg b.w. per month	4***	Australian Government (2005)
United States	2004	PCDDs and PCDFs	9.6 pgWHO-TE/kg b.w. per month*	47	EPA (2006)
Sweden	2006	PCDDs, PCDFs and DL-PCBs	1.30 pgWHO-TE/kg/b.w. per day**	16	Darnerud et al. (2006)
Italy	2006	PCDDs, PCDFs and DL-PCBs	2.28 pgWHO-TE/kg b.w.	11	Fattore et al. (2006)
France	2005	PCDDs, PCDFs and DL-PCBs	1.8 pgWHO-TE/kg b.w.	10	Tard et al. (2007)

* Lower bound approach, all groups.
** Medium bound approach.
*** Including eggs.

general scheme: known quantities of isotopically labelled analytes are added to the samples at the earliest possible stage of extraction to provide proper correction for analyte losses, then the test sample has to be homogenized and dehydrated as described for organochlorine pesticide analysis. The analytes of interest are extracted with a suitable organic solvent and the extract is purified by the use of sulfuric acid, as far as all the analytes of interest are resistant to acid treatment and this step allows a selective destruction of most of the interfering substances (i.e. fats) co-extracted with the target compounds. Due to the difference in concentrations between planar (PCDDs, PCDFs and non-ortho DL-PCBs) and non-planar analytes (mono-ortho DL-PCBs) and the presence of other co-extractive compounds resistant to clean-up procedure (i.e. chlorinated pesticides), fractioning steps are generally included during purification before instrumental analysis by HRGC-HRMS.

Polybrominated Diphenyl Ethers (PBDEs)

PBDEs are a group of 209 congeners, differing in the number of bromine atoms and in their position on two phenyl rings linked by an oxygen. Their nomenclature is identical to that of PCBs. PBDEs were first introduced into the market in the 1960 s and used as flame retardants to improve fire safety in various consumer products and in electronics. There are three types of commercial PBDE products, referred to as pentabromo- (Penta-BDE), octabromo- (Octa-BDE) and decabromodiphenyl ether (Deca-BDE), each product being a mixture of various PBDE congeners (Alaee, Arias, Sjödin, & Bergman, 2003). These chemicals are persistent and lipophilic, thus resulting in bioaccumulation in fatty tissues of organisms and in enrichment through the food chain (Law et al., 2003). The EU has prohibited the uses of Penta- and Octa-BDE (EU Directive, 2002), but these substances are still on the market in many regions of the world. In any case, a substantial reservoir of PBDEs exists in products that could release them to the environment.

Despite the fact that dietary intake is probably the main route of exposure to PBDEs for the general population (Schuhmacher, Kiviranta, Vartiainen, & Domingo, 2007; Schecter, Papke, Tung, Staskal, & Birnbaum, 2004), no MLs for PBDEs in food have been set by the EU yet. Tolerable daily intakes, due to the scarcity of data on human beings, have not been established yet.

Because they are not framed within national monitoring programs of contaminant residues in foods, PBDE occurrence data are basically recovered from intakes studies and generally are referred to the following congeners 28, 47, 99, 100, 153, 154, 183 and 209, as the most recurrent.

In the Netherlands, a granted national project revealed that oils and fats accounted for 25% of the total PBDE exposure, while milk, fish and meat contributed for 19, 13 and 11% of the intake. Average levels found in meat products, expressed as ng/g product (medium bound approach), were 0.152 for

beef (16% fat) and 0.273 for pork (26% fat) (de Mul et al., 2005); when converted on a fat basis, mean values resulted in 0.950 and 1.0 ng/g lipid base (lb) for beef and pig, respectively. An USDA (2005) study based on food consumption data recorded contamination values in pig meat for the selected eight PBDEs of 2.6 ng/g fat on average, with a rather wide range spanning from 0.190 to 16.3 ng/g fat. Such a range may indicate the presence of occasional sources capable of determining high levels of contamination. By contrast in beef, against a reported mean contamination of 0.250 ng/g fat, levels ranged from the detection limit to 0.880 ng/g fat (Huwe et al., 2005).

A total diet study recently carried out by the United Kingdom Food Safety Agency (UK FSA) estimated an upper bound dietary intake in adults of five PBDEs (PBDE 47, PBDE 99, PBDE 100, PBDE 153, PBDE 209) of 5.9 ng/kg b.w. per day, where meat products accounted for 68% contribution (FSA, 2006a). In Sweden (Darnerud et al., 2006), for an estimated intake of 50.9 ng/person/day (0.69 ng/kg b.w.), meat products contributed up to 14%. In Finland (Kiviranta, Ovaskainen, & Vartiainen, 2004), PBDE (six congeners) intake was estimated in 44 ng/day per person, where meat category (including eggs) represented 4% of the total.

As in the case of NDL-PCBs, PBDE determination in meat is limited to the of a small number of congeners used as indicators. The EFSA Scientific Panel on Contaminants in the Food Chain has recently recommended the inclusion of the following congeners in a European monitoring programme: PBDE 28, 47, 99, 100, 153, 154, 183 and 209 (EFSA, 2006) as the most frequently congeners found in food. Covaci, Voorspoels, and de Boer (2003) and Covaci et al. (2007) have recently reviewed the advances in the analysis of brominated flame retardants that in principle do not greatly differ from the approaches used for PCBs and "dioxins", with a Soxhlet or pressurized liquid extraction (PLE) in organic solvents, followed by a lipid removal via strong acidic step.

PBDEs are generally quantified by HRGC-MS operating in the NCI or EI mode (Thomsen et al., 2002). The characteristics of the GC system have to be properly selected to prevent the possible degradation for certain congeners (i.e. PBDE 209); useful indications on the most effective GC columns for PBDE congener-specific analysis can be found in the work of Korytár, Covaci, de Boer, Gelbin, and Brinkman (2005).

Polyfluorinated Alkylated Substances (PFAS)

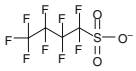
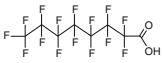
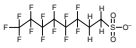
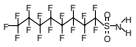
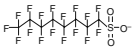
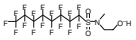
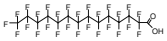
The polyfluorinated alkylated substances are compounds consisting of a hydrophobic alkyl chain of variable length (typically C_4–C_{16}) and a hydrophilic end group. The hydrophobic part may be fully or partially fluorinated: for instance, the "6:2" formula indicates that, in the C_8-chain, six carbons are fully fluorinated whereas the remaining two bear hydrogen atoms. When fully fluorinated the molecules are also called perfluorinated alkylated substances, whereas the

partially fluorinated ones, because of the telomerization production process, are also named telomers. The hydrophilic end group can be neutral or positively or negatively charged. The resulting compounds are non-ionic, cationic or anionic surface-active agents: due to their amphiphilic features, most of the perfluorinated compounds will not accumulate in fatty tissues as is usually the case with other persistent halogenated compounds.

PFAS can be widely found in the environment, resulting from anthropogenic sources as a consequence of industrial and consumer applications, including stain-resistant coatings for fabrics and carpets, oil-resistant coatings for paper products, fire-fighting foams, mining and oil well surfactants, floor polishes and insecticide formulations (Kannan et al., 2004) (Table 15.11).

At present, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the most investigated molecules in the environment due to their widespread occurrence, bioaccumulation and persistence. Relatively sparse

Table 15.11 Chemical structure of PFAS of potential interest

Compound	Molecular structure	Acronym	Molecular weight
Perfluorobutyl sulfonate (CAS 29420-49-3)		PFBS	299.21
Perfluorooctanoic acid (CAS 335-67-1)		PFOA	414.07
6:2 Fluorotelomer sulfonate (CAS 29420-49-3)		6:2 FTS	427.16
Perfluorooctane sulfonamide (CAS 754-91-6)		PFOSA	499.14
Perfluorooctyl sulfonate (CAS 2795-39-3; 1763-23-1 (acid))		PFOS	499.23
N-Methyl perfluorooctane sulfonamidoethanol (CAS 24448-09-7)		N-MeFOSE	557.23
Perfluorotetradecanoic acid (CAS 376-06-7)		PFTeDA	714.12

data are available on their presence in food of animal origin, mainly referred to fish and fishery products, a food category that at the present state of knowledge represents, with water, the main source of intakes (Hoff et al., 2003; Kannan et al., 2005; Yamashita et al., 2005; Haukas, Berger, Hop, Gulliksen, & Gabrielsen, 2007). Not-detectable residue in meat with respect to fish and eggs were reported in a total diet study carried out in the United Kingdom (FSA, 2006b). A very recent paper (Trudel et al., 2008) indicated levels in US and EU meat in the range from not detectable to 0.5 ng/g wet weight for PFOS and up to 1 for PFOA, respectively, and contaminations averaging between 0.2 and 0.3 ng/g.

Guidance values such as tolerable daily intake TDI have been recently proposed by UK and German health authorities: the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment recommended a TDI of 300 ng/kg b.w., for PFOS, and a TDI of 3000 ng/kg b.w. for PFOA (COT, 2006). The German Federal Institute for Risk Assessment (2006) derived a provisional TDI of 100 ng/kg b.w. for PFOS; the German Drinking Water Commission (2006) established a provisional TDI of 100 ng/kg b.w. for both PFOS and PFOA for the whole population, including infants and pregnant women. A duplicate diet study carried out in Germany (Fromme et al., 2008) indicated a median intake of 1.4 and 2.9 ng/kg. b.w. for PFOS and PFOA, respectively, with a negligible contribution from meat.

As far as no regulatory limits are in place for such category of contaminants in food, in the recent scientific literature, only few works deal with specific analytical methods for PFAS in meat. Due to the polarity of the molecules LC coupled to MS/MS detectors with an electrospray interface (ESI) is the instrumental technique of choice to identify and determine PFAS. Data can be acquired in the selected reaction monitoring (SRM) mode. Some non-polar fluorinated compounds, such as PFOSA, can be directly determined with HRGC-MS with positive chemical ionization (PCI). Tittlemier, Edwards, and Pepper (2006) described a liquid chromatography in tandem with mass spectrometry (LC-MS/MS) multi-residue method to analyse PFOS, PFOA and related compounds in composite samples of several foods (e.g. chicken, lamb, beef, pork) with a limit of determination (LD) ranging 0.5–1 ng/g fresh weight. However, the analytical procedure may recognize the following pitfalls described by Martin et al. (2004): (a) ion-suppression phenomena in ESI; (b) PFAS release from teflonated materials used in sampling handling, extraction and clean-up steps; (c) glassware capable to sequestrate PFAS when in aqueous solutions; (d) insufficient purity of the standards, for the presence of linear and branched isomers and a possible limited availability of ^{13}C labelled PFAS to be used as ISs for quantification; (f) reference materials not always available.

To limit as much as possible the external contamination of the samples, it is recommended to limit the extraction and clean-up procedures to the essential steps. However, such limitation may affect quantitative recoveries and method selectivity, thus leading to possible ion-suppression phenomenon during the instrumental acquisition of data (Powley, George, Ryan, & Buck, 2005). Effective procedures consist of a sample extraction with alkalinized methanolic

solution followed by a weak anionic exchange solid-phase clean-up as described by Taniyasu et al. (2005). Berger and Haukas (2005) proposed to use a polar solvent extraction followed by a clarification of the extract via centrifugation and a selective filtration at 3000 nominal molecular weight limit cut-off, prior to instrumental analysis.

Conclusions

Due to their intrinsic properties, POPs represent a scientific-based challenge for meat quality and safety issues, for both local and worldwide consumption. Meat-producing animals may represent both the sentinels of the effectiveness of environmental policies focused on reducing the release of the aforesaid contaminants, and, at the same time, one of the relevant sources of intake for consumers. Such a double aspect stresses the relevance of the implementation of national monitoring plans and of the parallel adoption of hazard analysis of critical control points (HACCP) schemes, possibly at farm level, as the most preventive and cost-effective actions to reduce the exposure and the subsequent bioaccumulation in food-producing animals (a "farm-to-fork approach"). The claimed effectiveness of such preventive policy can be cross-checked through the assessment of population intakes, with sampling and analytical strategies targeted on what is really eaten, reflecting dietary, cooking and food dressing habits of the population and sensitive groups (a "from-fork-to-farm approach"). The reliable information obtained by matching the occurrence of contaminants and intakes could help risk managers and meat stakeholders to take the best appropriate and scientifically supported risk management and risk communication actions.

Glossary

AL	action level
AOAC	Association of Official Analytical Chemists
ASE	accelerated solvent extraction
COT	Committee on Toxicity of Chemicals in Food
DD	duplicate diet
pp′-DDT	1,1,1-trichloro-2,2-*bis*(4-chlorophenyl)ethane
Deca-BDE	decabromodiphenyl ether
DL-PCB	dioxin-like polychlorinated biphenyls
EC	European Commission
ECD	electron capture detector
EFSA	European Food Safety Authority
EI	electron impact
EPA	Environmental Protection Agency
ESI	electrospray interface
EU	European Union

FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FSA	Food Safety Agency
GPC	gel permeation chromatography
HACCP	hazard analysis of critical control points
alpha-HCH	*alpha*-hexachlorocyclohexane
beta-HCH	*beta*-hexachlorocyclohexane
HPLC	high-performance liquid chromatography
HRGC	high-resolution gas chromatography
HRMS	high-resolution mass spectrometry
IARC	International Agency for Research on CancerIS internal standard
LC	liquid chromatography
LRMS	low-resolution mass spectrometry
ML	maximum level
MRL	maximum residue limit
NDL-PCB	non-dioxin-like polychlorinated biphenyl
Octa-BDE	octabromodiphenyl ether
PBDE	polybrominated diphenyl ether
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
Penta-BDE	pentabromodiphenyl ether
PFAS	polyfluorinated alkylated substances
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PLE	pressurized liquid extraction
POP	persistent organic pollutant
SCCP	short-chained chlorinated paraffin
SCF	Scientific Committee on Food
SFE	supercritical fluid extraction
SPE	solid-phase extraction
TDI	tolerable daily intake
TDS	total diet study
TMI	tolerable monthly intake
TWI	tolerable weekly intake
UNEP	United Nations Environment Programme
USDA	United States Department of Agriculture
WHO	World Health Organization

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Chapter 16
Real-Time PCR Methods for Detection of Foodborne Bacterial Pathogens in Meat and Meat Products

Marta Hernández, Flemming Hansen, Nigel Cook, and David Rodríguez-Lázaro

Introduction

As a consequence of the potential hazards posed by the presence of microbial pathogens, microbiological quality control programmes are being increasingly applied throughout the meat production chain in order to minimize the risk of infection for the consumer. Classical microbiological methods to detect the presence of microorganisms, involving enrichment and isolation of presumptive colonies of bacteria on solid media, and final confirmation by biochemical and/or serological identification, although remaining the approach of choice in routine analytical laboratories, can be laborious and time consuming. The adoption of molecular techniques in microbial diagnostics has become a promising alternative approach, as they possess inherent advantages such as shorter time to results, excellent detection limits, specificity and potential for automation. Several molecular detection techniques have been devised in the last two decades, such as nucleic acid sequence-based amplification (NASBA) (Cook, 2003; Rodriguez-Lazaro, Hernandez, D'Agostino, & Cook, 2006) and loop-mediated isothermal amplification (Notomi et al., 2000), but the one which has undergone the most extensive development as a practical food analytical tool is the polymerase chain reaction (PCR) (Hoorfar & Cook, 2003; Malorny, Tassios, et al., 2003). A considerable number of methods, based on PCR, for detection of pathogens in meat have been published, and there are several methods marketed commercially. This chapter will focus only on open-formula methods published in the scientific literature. Due to their transparency, such methods have the potential for adoption as international standards (Hoorfar & Cook, 2003). Furthermore, focus is given only to real-time PCR-based methods. Although PCR is a simple, versatile, sensitive,

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specific and reproducible assay (Malorny, Tassios, et al., 2003; Scheu, Berghof, & Stahl, 1998), in its conventional format it does not allow the quantification of the bacterial load. Real-time PCR represents a significant advance as it can allow quantification of the starting number of target molecules in the reaction (Heid, Stevens, Livak, & Williams, 1996); moreover it is faster, less prone to cross-contamination problems and has the potential for automation.

PCR: Principles and Applications

Kleppe et al. described the principles of polymerase chain reaction (PCR) for first time in 1971. But it was in 1985, with the introduction of a thermostable DNA polymerase, that Saiki et al. published the first experimental data (Saiki et al., 1985, 1988). The discovery of PCR is attributed to a collaborator of Saiki, Kary B. Mullis, laureate with the Nobel Prize in Chemistry in 1993. This technique has been applied in different areas due to its versatility, specificity and sensitivity. Accordingly, PCR has been successfully used for microorganism identification (Rodriguez-Lazaro et al., 2007). It is an exponential amplification of a DNA fragment, and its principle is based on the mechanism of DNA replication in vivo: dsDNA is denatured to ssDNA, duplicated, and this process is repeated along the reaction.

The development of real-time (RTi-) PCR in 1996 represents a significant advance as it allows monitoring of the synthesis of new amplicon molecules by using fluorescence during the cycling that can be used to quantify the initial amounts of template DNA molecules. Data are therefore collected throughout the PCR process, not only at the end of the reaction (as occurs in conventional PCR). Fluorescence can be produced by an unspecific detection strategy independent of the target sequence using fluorescent molecules when bound to dsDNA (e.g. ethidium bromide, YO-PRO-1 or SYBR Green I) or by sequence-specific fluorescent oligonucleotides (hydrolysis and hybridization probes). The hydrolysis probes are cleaved by 5′–3′ exonuclease activity during the elongation phase of primers. One of the most used are the TaqMan® probes that are double-labelled oligonucleotides with a reporter fluorophore at the 5′ end and a quencher internally or at the 3′ end. The quencher dye absorbs the fluorescence of the reporter dye due to its proximity, which permits the physical phenomenon defined as "fluorescence resonance energy transfer" (FRET). In contrast to hydrolysis probes, hybridization probes are not hydrolysed during PCR. The fluorescence is generated by a change in its secondary structure during the hybridization phase, which results in an increase in the distance separating the reporter and the quencher dyes. The most relevant hybridization probes are those containing hairpins (Molecular Beacons, Scorpion primers, etc.) and FRET hybridization probes. In both type of probes, the signal generated is proportional to the amount of specific amplicon produced during the reaction and is detected by a sensitive charge-coupled device (CCD). The

fluorescence is typically represented by the software of analysis as an amplification curve (amplification plot) with three different phases. The first is called the *initiation phase*, and the emitted fluorescence cannot be distinguished from the baseline (usually between 3 and 15 cycles). Then, it is produced an *exponential* or *log phase* in which fluorescence increase up to the *plateau phase*, where the reagents are exhausted, and no increase in fluorescence is observed. Only at the beginning of the exponential phase is quantification possible because amplification is most efficient, and therefore quantification is less affected by reaction-limiting conditions. The analysis system allows to establish a threshold (can be fixed automatically) to which corresponds a cycle value called the *threshold cycle* or C_T, or *crossing point* C_P, which corresponds to the cycle at which a statistically significant increase in fluorescence is first detected. Therefore, the number of cycles needed for the amplification-associated fluorescence to reach a specific threshold level of detection (the C_T or C_P value) is inversely correlated to the amount of nucleic acid that was in the original sample, that is, the higher the template copy number, the shorter the time to reach C_T or C_P. Thus, the real-time PCR is the ideal tool to quantify the presence of a sequence by interpolation of the resulting C_T or C_P value in a linear standard curve of values obtained from serially diluted known-amount standards over more than five orders of magnitude.

Critical Features of a PCR-Based Method

Among the many published methods there are some with little potential for routine implementation and others which have been specifically designed to fulfil this final purpose. The main features which an ideal molecular analytical method should possess are defined high performance characteristics, efficient sample preparation and appropriate controls. Each of these required features will be discussed in more detail below.

The International Standard Performance Characteristics

Among different molecular-based techniques currently available, only PCR has been considered for the International Bodies of Normalisation as an analytical tool up to now, and the unique in which these institutions have been working with. In this scenario, the Technical Committees CEN/TC 275 "Food analysis – Horizontal methods" of the European Committee for Standardization (CEN) working group WG 6 "Microbial contamination" and ISO/TC "Food products" subcommittee SC9 "Microbiology" of the International Organization for Standardization (ISO) have developed the International Standard ISO 22174, *Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens – General requirements and*

definitions (Anonymous, 2005b), in which principal criteria and parameters for PCR performance as a diagnostic tool are defined. ISO 22174 defines specificity as "the capacity to exclusively recognise the target to be detected, distinguishing it from similar substances and impurities". In other words, a fully selective PCR should consistently detect the desired target and be unable to detect non-targets. In addition, ISO 22174 also defines the limit of detection (LOD) of a PCR-based method as "the lowest concentration or content of the target microorganism per defined amount of food matrix that can be consistently detected under the experimental condition defined in the method". This limit of detection may also been defined as analytical sensitivity (Rodriguez-Lazaro, Pla, Scortti, Monzo, & Vazquez-Boland, 2005). The ideal PCR assay should be fully specific and possess an excellent analytical sensitivity, e.g. be able to detect 10^0–10^1 targets per reaction.

There are some other critical analytical parameters for food analysts: accuracy, precision and robustness. Accuracy describes the veracity of the test results (Skoog & Leary, 1992) and can be defined as closeness of agreement between a test result and the accepted reference value (Anonymous, 1993; Paoletti & Wighardt, 2002). Similar terms are also trueness and relative accuracy. The first can be defined as closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (Anonymous, 1994; Thompson, Ellison, & Wood, 2002), and the second as the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (Anonymous, 2003a; Hoorfar & Cook, 2003). Precision describes the reproducibility of the test results (Skoog & Leary, 1992) and can be defined as the closeness of agreement between independent test results obtained under stipulated conditions of repeatability and reproducibility (Anonymous, 1993; Thompson et al., 2002). Repeatability is the closeness of agreement between successive and independent results obtained by the same method on identical material, under the same repeatability conditions (apparatus, operator, laboratory and short intervals of time) (Anonymous, 2003a), and reproducibility is the closeness of agreement between single test results on identical test material using the same method and obtained by operators in different laboratories using different equipment (that is reproducibility conditions) (Anonymous, 2003a). Finally, robustness is the reproducibility by other laboratories using different batches and brands of reagents and validated equipment (Hoorfar & Cook, 2003).

Sample Preparation

The efficiency and performance of molecular methods can be negatively affected by some food components (Rådström, Knutsson, Wolffs, Dahlenborg, & Löfström, 2003; Rodriguez-Lazaro & Hernandez, 2006; Rossen, Nøskov,

Holmstrøm, & Rasmussen, 1992). These inhibitors, by interfering with nucleic acid amplification, can lead to underestimation of the bacterial load, or to false-negative results. Thus, efficient sample preparation is crucial for the performance of molecular-based methods and is vital to the implementation of molecular methods as routine diagnostic tools.

Molecular-based methods such as those based on PCR or NASBA work with small volumes (10–50 μl), but the size of the food sample required for realistic microbiological analysis is several orders of magnitude greater than this (e.g. 25 g). Bacterial pathogens need in many instances only to be present in low numbers in a foodstuff to pose a hazard to the consumer. Therefore, to have an efficient molecular-based method for detection of microbial pathogens in a food sample, this potentially low number of targets must be delivered into the reaction. Consequently, the target pathogen or its nucleic acid must be concentrated out of the foodstuff and be contained in a small volume for addition to the reaction components. With bacterial targets, the principal approaches to this are enrichment of the cells by incubating the food sample in a nutrient broth thus increasing the number of targets by volume, and chemical extraction of target nucleic acids and concentration into a small volume. With the use of an enrichment step prior to bacterial nucleic acids extraction, two important purposes can be achieved; i.e. the concentration of target bacteria and the dilution of inhibitory substances that can affect the subsequent analytical steps. Furthermore, as only viable bacterial cells can grow, an enrichment step can be adapted for viability studies, and the use of an enrichment step prior to a molecular-based detection (specially in the case of NASBA) may be also a safeguard against false-positive results in a viability study caused by residual nucleic acids (i.e. DNA for PCR or RNA for NASBA) in dead cells (Cook, 2003). However, when enrichment is applied prior to PCR, the accuracy of this approach will depend on the background of DNA form dead cells in the sample. This methodology is very simple and cost-effective; however, the selection of the appropriate enrichment medium will depend on the complexity and homogeneity of the food samples and the accompanying microbiota.

Controls

A series of controls is necessary to assess the analytical performance of a method and to ensure that the correct interpretation of the results is made. The International Standard ISO 22174 describes the principal controls that must be added to any PCR-based method to assess its correct analytical performance, and they are summarized in Table 16.1.

The Internal Amplification Control (IAC) is a non-target nucleic acid sequence present in every reaction, which is co-amplified simultaneously with the target sequence (Hoorfar, Cook, et al., 2003; Hoorfar, Malorny, et al., 2004). In a reaction without an IAC, a negative signal can mean that there was no target

Table 16.1 Analytical controls for assessing the correct performance of a PCR-based method

Process controls
Processing positive control (PPC). *A sample spiked with the bacterial target and processed by the method. A positive signal should be obtained, showing that the entire analytical process was correctly performed*
Processing negative control (PNC). *A known uncontaminated sample, which is processed by the method. A negative signal should be obtained, showing that no contamination by the target bacterium or its nucleic acid has occurred during the process*
Negative extraction control or extraction blank (NEC). *A control carried through all the steps of the DNA extraction procedure, but not contained within a test sample (e.g. an aliquot of sterile H_2O). This control is termed an "amplification control" in the ISO standard 22174*
Environmental control (EC). *A PCR tube containing the master mixture left open in the PCR setup room to detect possible contaminating nucleic acids in the environment. This is a useful control for troubleshooting if a positive signal appears in the PNC. However, this control is not included in the ISO Standard 22174*
Amplification controls
Positive PCR control. *A PCR to which has been added a known amount of target nucleic acid. A positive signal indicates that the reaction was performed correctly*
Negative PCR control *(or no template control – NTC – or reagent control or blank). A reaction which includes all reagents but not the target nucleic acids. Usually, water is added instead of the template. A negative signal shows that the PCR mastermix was not contaminated*
External amplification control (EAC). *An aliquot of a solution of control DNA, containing a defined quantity or copy number, added to an aliquot of the extracted sample's nucleic acid, and analysed in a separate reaction tube. A positive signal indicates that the sample's nucleic acid extract did not contain any inhibitory substances)*
Internal amplification control (EAC). *A DNA added to each reaction in a defined amount or copy number, which serves as an internal control for amplification. When there is no target signal and no IAC signal, this means that the PCR has failed*

Adapted from ISO 22174, Anonymous (2005b), Hoorfar and Cook (2003) and Rodriguez-Lazaro et al. (2007).

sequence present in the reaction. *But* it could also mean that the reaction had failed, due to malfunction of equipment, incorrect reaction mixture, poor enzyme activity or the presence of inhibitory substances. In a reaction with an IAC, a control signal will always be produced when there is no target sequence present. When no IAC signal is observed, this means that the reaction has failed. The IAC is an absolutely essential feature, and any method which does not contain one has no practical value in actual food analysis, since without an IAC negative results cannot be accepted as unambiguously signifying that the original sample did not contain the target microorganism. This review therefore will focus only on those published methods which include this control.

Quantitative Capacity

Quantification of the bacterial loads can provide an important tool for the microbial risk assessment. Risk assessment can be defined as the quantitative or

qualitative determination of a risk related to a concrete situation and a recognized hazard. It is one of the pieces of the global process of risk analysis (which also includes risk management and risk communication), and it is one of the instruments used for food regulators and policy decision makers at both national and international levels in food safety. However, from a meat industry point of view, there is only a very limited need for quantification in pathogen testing in that sector: currently only for *Listeria monocytogenes* in meat products. In addition, most of the available methods still need a short enrichment period, making the value of the final quantification obtained by PCR very doubtful. However, one of the most important challenges and current trends in molecular methodology in food microbiology is the development of new and improvement of the current pre-amplification treatments in order to guarantee a direct bacterial nucleic acid extraction from the food sample and therefore the effective direct application of the molecular methods without any enrichment period (Malorny et al., 2003b; Rodriguez-Lazaro et al., 2007). In this analytical scenario, the capacity of a real-time PCR method to accurately determine the number of targets present in the sample depends on the linearity and efficiency of the PCR. Linearity is the ability of the method to generate results proportional to the amount of target molecule present in the sample (Rodriguez-Lazaro et al., 2005). Efficiency is the capacity of the PCR to duplicate the amplicon molecules in each cycle (Rodriguez-Lazaro et al., 2005).

Real-Time PCR Methods for the Principal Foodborne Pathogens in Meat and Meat Products

This section provides brief descriptions of the currently available real-time PCR-based methods for detection of the most relevant foodborne pathogens in meat and meat products: *Salmonella*, *Campylobacter*, *Shiga-toxin-producing Escherichia coli* and *L. monocytogenes*. Other important microbial pathogens in meat and meat products such as *Yersinia enterocolitica* and *Staphylococcus aureus* do not have specific open-formula real-time PCR methods including IAC, which is a principal control that can be included in each analytical method, and therefore they will not be discussed in this section. Table 16.2 summarizes the principal analytical features of each described method.

Salmonella

Malorny et al. (2004) developed a robust real-time PCR method for detection of *Salmonella enterica* and *S. bongori* in different meat products. The target of the real-time PCR assay was the *ttrRSBCA* gene, required for the tetrathionate respiration in this bacterium, and which is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. The platform used by the authors was the

Table 16.2 Real-time quantitative PCR methods for the principal foodborne pathogens in meat and meat products

Bacterium	Target sequence	Matrix	Sample preparation	Limit of detection (LOD)	References
Salmonella	*ttr*RSBCA gene	Chicken carcass, minced meat	Enrichment	≈ 1 CFU/25 g food sample	Malorny et al. (2004)
	invA gene	Minced beef meat	Enrichment	≈ 1 CFU/25 g food sample	Perelle, Dilasser, Malorny, et al. (2004)
	Prot6e and *invA* genes	Chicken carcass	Enrichment	10 genome equivalents/ reaction	Malorny et al. (2007)
	invA and *invE* genes	Meat and meat products	Enrichment and immunomagnetic separation	≈ 1 CFU/25 g food sample	Notzon et al. (2006)
	invA gene	Minced meat	Enrichment	≈ 1 CFU/25 g food sample	Josefsen et al. (2007)
	invA gene	Beef, chicken and pork	Enrichment	≈ 1 CFU/25 g food sample	Bohaychuk et al. (2007)
Campylobacter	16S RNA gene	Pork and chicken carcasses	Enrichment	10 CFU/ml carcass rinse	Perelle, Josefsen, et al. (2004)
	16S RNA gene	Chicken carcass	Enrichment	≈ 1 CFU/25 g food sample	Josefsen et al. (2004)
	16S RNA gene	Chicken carcass	Enrichment	≈ 1 CFU/25 g food sample	Krause et al. (2006)
E. coli	*stx1* and *stx2* genes	Beef meat	Enrichment	–	Perelle et al. (2007)
	stx1 and *stx2; eaeO26* and *eaeO111*, and wzt_{O91} genes	Bovine and veal mincemeat	Enrichment	10 CFU/25 g food sample	Stefan et al. (2007)
L. monocytogenes	*hly* gene	Raw pork, cooked ham, fermented pork sausage, frankfurter sausage	Filtration	100 CFU/g	Rodriguez-Lazaro et al. (2004)
	hly gene	Raw pork, cooked ham, fermented pork sausage, frankfurter sausage	Direct PCR	$3' 10^5$ CFU/g	Rodriguez-Lazaro et al. (2005)
	ssrA gene	Hotdog, sliced ham, pate and sliced turkey	Enrichment	1–10 genome equivalents/ reaction	O'Grady et al. (2008)

DNA Engine Opticon 2 System (MJ Research, South San Francisco, USA). This method was able to identify 110 *Salmonella* strains correctly, and not to detect 87 non-*Salmonella* strains. The authors inoculated a 100-ml aliquot of a whole-chicken carcass rinse at four levels (0, 5, 26 and 474 CFU per 100 ml of rinse) with *S. enterica* and incubated at 37°C for 20 h prior to testing. In addition, they also artificially contaminated minced meat with *Salmonella* at five levels (0, 1–5, 5–10, 10–20 and 20–100 CFU/25 g). DNA was extracted from 1-ml aliquots of the resulting cultures, by Chelex-100 resin (Biorad, Munich, Germany). In parallel, a classical microbiological method was performed according to international standard ISO 6579:2003, which is the internationally accepted culture method to detect *Salmonella* in foodstuffs (Anonymous, 2003b). The results obtained in all the artificial contamination levels of 100-ml whole-chicken carcasses rinses and 25 g of minced meat resulted in 100% agreement of *Salmonella* detection between the traditional and the real-time PCR methods: i.e. the diagnostic sensitivity (the proportion of culture-positive samples that test positive in the PCR assay) was 100%, and the diagnostic specificity (the proportion of culture-negative samples that test negative in the PCR assay) was 100%. Finally a total of 43 potentially naturally contaminated meat samples (23 whole-chicken carcass rinses, and 20 samples of minced meat from pig and cattle) were analysed by the traditional culture method according to international standard ISO 6579:2003 and the real-time PCR assay. The overall relative diagnostic sensitivity, specificity and accuracy were 100%.

Malorny and co-workers had also previously devised a conventional PCR assay for *Salmonella* based on targeting sequences of the *invA* gene and validated its analytical accuracy in two collaborative trials (Malorny, Hoorfar, Bunge, & Helmuth, 2003; Malorny, Hoorfar, Hugas, et al., 2003). Perelle, Dilasser, Malorny, et al. (2004) adapted this assay to real-time format using the LightCycler platform (Roche Diagnostics). They evaluated the selectivity of the new real-time PCR method using 84 *Salmonella* and 44 non-*Salmonella* strains, obtaining that the real-time PCR assay was 100% selective. Finally, they artificially contaminated 25 g of minced beef meat with different concentrations of *Salmonella* (0, 1–5, 5–10, 10–20, 20–200 CFU/25 g), and 10-fold diluted them in buffered peptone water, and subsequently they were incubated 18 h at 37°C. One millilitre of enrichment was used for the bacterial DNA extraction using the InstaGene Matrix (Bio-Rad Laboratories, Germany). Simultaneously they analysed the enrichments by the standard culture-based method ISO 6579 (Anonymous, 2003b). There was 100% agreement between the results obtained by the two methods.

Malorny and co-workers have developed and in-house validated a duplex TaqMan PCR method for the detection of both *Salmonella* spp. and *S. enterica* subspecies *enterica* serovar Enteritidis in whole-chicken carcass rinses (Malorny, Bunge, & Helmuth, 2007). The duplex real-time PCR method targeted the Prot6e gene located on the *S.* Enteritidis-specific 60-kb virulence plasmid for the specific detection of *S.* Enteritidis, and the gene *invA* for the detection of *Salmonella* spp. For each PCR assay (Prot6e and *invA*) the other Q-PCR run in duplex worked as non-competitive IAC. The duplex Q-PCR method was 100%

inclusive for the *Salmonella*-specific *invA* real-time PCR (i.e. detection of all 193 *Salmonella* strains), and 95% for the *S*. Enteritidis-specific Prot6e PCR (i.e. 75 out of 79 *S*. Enteritidis strains were positively detected). From these four negative strains, two showed a plasmid profiling that differed in size (104 and 69 kb) from the common *S*. Enteritidis 60-kb virulence plasmid. The other two strains did not possess any plasmid. On the other hand, the *S*. Enteritidis-specific Prot6e real-time PCR was 100% exclusive (no detection of the 114 non-Enteriditis *Salmonella* strains). The duplex method detected consistently 100 *Salmonella* spp. or *S*. Enteritidis genome equivalents and 10 genome equivalents in 83% of the PCR replicates. For the use of the duplex method in poultry meat, 50 ml of whole-chicken carcass rinses prepared according to the ISO 6887-2 (Anonymous, 2003c) using ml 1% (w/v) buffered peptone water was artificially contaminated with decreasing load levels of *S*. Enteritidis strains. In addition, chicken carcass rinses of 25 potentially naturally contaminated chickens were also compared by the traditional culture method according to ISO 6579:2003 (Anonymous, 2003b) and the duplex real-time PCR method. The samples were incubated for 18–20 h at 37°C without shaking, and 1-ml aliquots were used for DNA extraction using Chelex-100 resin (Bio-Rad, Munich, Germany). The PCRs were run in the DNA Engine Opticon 2 System (MJ Research). The *Salmonella* detection between the traditional culture and the PCR method resulted in 100% agreement: i.e. non-inoculated and 20 potentially naturally contaminated carcass rinses were negative whereas all artificially inoculated samples and five naturally contaminated carcass rinses were positive by both methods. Consequently, the overall relative sensitivity, specificity and accuracy were 100%. Two out of the five positive samples for *Salmonella* (both for traditional culture method and *invA* real-time PCR) were also positive for the Prot6e real-time PCR assay and were confirmed by serotyping to be *S*. Enteritidis.

Notzon, Helmuth, and Bauer (2006) developed an immunomagnetic separation (IMS)–real-time PCR method for detection of *Salmonella* in meat products which provided a definitive result after 13 h. Pork cutlets purchased from a local butchery were artificially contaminated with 1, 10 and 100 CFU/25 g for determination of the detection limit of the IMS–real-time PCR method. The same study investigated 491 naturally contaminated meat samples comprising unpackaged meat purchased from local butcheries and packaged meat purchased from local supermarkets using an immunomagnetic separation (IMS)–real-time PCR assay, and the results obtained were compared with those obtained using the German reference microbiological method for the detection of *Salmonella*. Meat samples ($\approx$ 25 g) were diluted (1:10) in buffered peptone water (BPW), homogenized and subsequently incubated at 37°C for 6 h. A 20-ml aliquot was centrifuged at 91 $\times$ *g* for 2 min, and 10 ml of the supernatant was used for the IMS using the Dynabeads anti-*Salmonella* (Dynal, Oslo, Norway). Subsequently, the bead-bacteria complexes were digested with proteinase K, and the extracted DNA was purified using the HighPure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany). PCRs were run in a LightCycler instrument (Roche), and the PCR method targets

were the *invA* and *invE* genes. The PCR method detected all samples artificially contaminated with 10 and 100 *Salmonella* CFU/25 g, and 10 out of 12 samples with 1 CFU/25 g; however, the two samples not detected were also negative using the German reference method. The diagnostic specificity (the proportion of culture-negative samples that test negative in the PCR assay) and diagnostic sensitivity (the proportion of culture-positive samples that test positive in the PCR assay) of the method was 80 and 100%, respectively, for artificially contaminated samples, and 99.3 and 83.7%, respectively, for natural samples. The relative accuracy was higher than 90% with both natural and artificially contaminated samples, and the concordance index k (statistical accordance between methods) was also higher than 0.85.

Josefsen, Krause, Hansen, and Hoorfar (2007) brought the time required for enriching samples prior to PCR down to 8 h – while still mediation of growth of low numbers of *Salmonella* cells to levels high enough to be detected – by optimizing critical steps in the whole method. These modifications were (i) increasing the volume of broth taken for nucleic acid extraction (1–5 ml); (ii) increasing the amount of paramagnetic particles in the DNA extraction (from 60 to 90 μl); and (iii) increasing the volume of nucleic acid extract used in the PCR (from 5 to 20 μl). Finally, the optimized method could be performed in its entirety in 12 h, with a relative accuracy of 99% compared to a standard culture-based detection method (Anonymous, 1999).

Bohaychuk, Gensler, McFall, King, and Renter (2007) tested artificially and naturally contaminated samples of beef, chicken and pork by an enrichment-real-time PCR method. The target of the assay was the *invA* gene, and the platform was the LightCycler (Roche Diagnostics, Mannheim, Germany). The specificity of the real-time PCR assay was 100% as it was able to classify correctly 77 bacterial isolates (51 *Salmonella* and non-*Salmonella* 26 isolates). Carcass-rinse swabs (from beef, pork and chicken) were artificially contaminated with decreasing loads of *Salmonella*, incubated in buffered peptone water at 35°C for 24 h, then aliquots of the resulting culture were transferred to both tetrathionate broth and Rappaport-Vassiliadis' broth and incubated each at 35°C for 24 h. Aliquots of each subsequent culture were then combined and cells pelleted by centrifugation before being subjected to DNA extraction. The original cultures were taken through the full conventional detection and identification procedures (plating, biochemical and serological testing). The results of each testing procedure were compared. The diagnostic sensitivity and specificity were 100%. Finally, 874 carcass-rinse swabs were evaluated as field samples. For them, the diagnostic sensitivity was 100%, and the diagnostic specificity was 91–98%.

Campylobacter *spp.*

Lübeck, Wolffs, et al. (2003) devised a primer set, targeting 16S RNA gene sequences, which was highly and robustly selective for the three thermophilic

campylobacters *C. coli*, *C. lari* and *C. jejuni* in conventional PCR (Lübeck, Cook, Wagner, Fach, & Hoorfar, 2003). These primers were subsequently incorporated in real-time PCR assays using a variety of instrument systems such as Roche LightCycler (Perelle, Josefsen, et al., 2004), RotorGene and ABI-PRISM (Josefsen, Jacobsen, & Hoorfar, 2004). Perelle and co-workers evaluated the selectivity of the LC-PCR method testing 39 *Campylobacter* and 9 non-*Campylobacter* strains. The results that they obtained indicated that the method was highly specific, giving cross-reactivity with only one strain of *C. upsaliensis*. To allow the detection of thermophilic campylobacters in pork carcass swabs and chicken rinse, 25-ml samples were treated prior to PCR by incubation in 225 ml Bolton Broth for 20 h at 42°C, then taking 1 ml of the resulting culture for nucleic acid extraction. The sensitivity of the LC-PCR assay developed by Perelle and co-workers, evaluated in 32 spiked poultry-rinse or pork carcass-swab samples, was determined at 10 CFU/ml carcass rinse. In the case of the RotorGene and ABI-PRISM methods developed by Josefsen and co-workers, the meat samples were spiked with 0, 1–10, 10–100 and 100–1000 CFU/250 ml. PCR detection was possible on both instruments at all spiking levels. In addition, Josefsen and colleagues evaluated 66 presumably naturally contaminated chicken rinse samples, and thermotolerant campylobacters were found in 40 samples by the culture-based method and with the RotorGene system, whereas the ABI-PRISM gave 39 positive responses.

Krause et al. (2006) proceeded towards the goal of implementation of PCR-based campylobacter PCR method, by organizing a multicentre collaborative trial involving nine Danish laboratories analysing enriched samples from various poultry-associated matrices, including chicken neck skin, where the enrichment broth had been spiked with *C. jejuni* at three levels (unspiked, 1–10 and 10–100 CFU/100 ml). The real-time PCR method used was that developed by Josefsen and co-workers (2004) (Corbett Research, Australia), with the only modification of the addition of a PCR facilitator, glycerol. The participants analysed the samples using the real-time PCR-based protocol on a variety of instrument systems. For each spiking level, the results from the chicken neck skin analysis gave 100% accuracy at all levels. The method was subsequently further validated at a major Danish abattoir, on cloacal samples on poultry on a flock basis. The method has now been implemented in routine use by several Danish poultry producers (Krause et al., 2006).

Shiga-Toxin-Producing Escherichia coli

Developing a strategy for screening of bovine products for the presence of common serogroups of Shiga-toxin-producing *E. coli* (STEC), Perelle, Dilasser, Grout, and Fach (2007) tested meat samples by a method based on PCR-ELISA. Twenty-five-gram samples were cut up and homogenized in a blender, then placed in 225 ml of nutrient broth and incubated at 37°C overnight. A 1-ml

aliquot was then taken for DNA extraction. The PCR primers targeted conserved *stx* gene sequences (Fach, Perelle, Dilasser, & Grout, 2001). After alkaline denaturation of the PCR products, they were added into streptavidin-coated microtiter plates where they were captured by biotin-labelled specific oligonucleotides. Following hybridization with a digoxigenin (DIG)-labelled oligonucleotide probe, a colorimetric signal was induced by addition of a peroxidase anti-DIG conjugate. PCR-ELISA-positive nucleic acid extracts were then examined by a multiplex real-time PCR screening for *E. coli* O26, *E. coli* O103, *E. coli* O111, *E. coli* O145 and *E. coli* O157 (Perelle, Dilasser, Grout, & Fach, 2004). Finally, positive multiplex assay samples were further tested by uniplex PCR to identify the exact O-serogroups and by an *stx*-typing 5′-nuclease assay (Perelle, Dilasser, Grout, et al., 2004).

Stefan et al. (2007) developed a real-time PCR method for the specific detection of Shiga-toxin-producing *E. coli* in minced meat products. This real-time PCR method included PCR primers and probes targeting the Shiga toxin genes *stx1* and *stx2* encoding the Shiga toxin 1 and Shiga toxin 2, respectively (Sharma, 2002), the eae_{O26} and eae_{O111} genes encoding the intimin of *E. coli* O26 and *E. coli* O111, respectively (Sharma, 2002), the wzt_{O91} gene encoding the "putative flippase" (Perelle, Dilasser, Grout, & Fach, 2002) and the $ihp1_{O145}$ and $ihp1_{O147}$ genes encoding the "inserted hypothetical protein 1" of *E. coli* O145 and *E. coli* O147, respectively (Perelle et al., 2002; Perelle, Dilasser, Grout, & Fach, 2003). The repeated element 1711 B (Walker et al., 2003) was used as non-competitive IAC. One hundred and six bovine and veal minced meat natural samples from different local supermarkets in Italy and artificially contaminated minced meat samples with decreasing levels of *E. coli O157* (500–1000 to 5–10 CFU/25 g of sample) were investigated. Twenty-five grams of each sample was diluted (1:10) in 225 ml of modified EC medium (Biokar Diagnostics, Alonne, France) containing 20 mg/l of novobiocin, homogenized for 2 min and then incubated at 37°C overnight without shaking. One-millilitre aliquots were used for the DNA extraction using the Nucleospin™ tissue kit (Machery Nagel, Duren, Germany). PCRs were carried out on an ABI Prism SDS 7900™ cycler (Applied Biosystems, Foster City, USA). The method (when targeting the *stx* and $ihp1_{O147}$ genes) robustly detected 10 STEC cells in 25 g of artificially contaminated mincemeat. From the 106 natural samples, only one was positive (<1%) for the *stx1* PCR, but it was negative for the *E. coli* O26-, O91-, O111-, O145- and O157-specific PCRs. Furthermore, the analytical sensitivity was compared with an enzyme-linked fluorescent assay (ELFA) method. Both methods worked equally for contamination levels down to 50 bacterial cells/25 g, but the real-time PCR method showed a lower limit of detection (10 STEC cells/25 g).

Listeria monocytogenes

A real-time PCR method for the quantitative detection of *L. monocytogenes* in meat products was developed by Rodriguez-Lazaro et al. (2005). The q-PCR

method was based on a previous developed PCR assay developed by the same research laboratory (Rodriguez-Lazaro, Hernandez, et al., 2004) and targeted the *hly* gene encoding the listeriolysin O (Mengaud et al., 1988). Twenty-five-gram samples of raw pork meat, fermented pork sausage, cooked ham and frankfurter sausage were artificially contaminated with decreasing amounts (approximately 3×10^7, 3×10^6 and 3×10^5 CFU/g) of *L. monocytogenes* CTC1010, and immediately homogenized in 1:10 (wt/vol) in BPW. One microlitre of the homogenate, without any DNA isolation treatment, was directly added to the TaqMan-based Q-PCR mixture (Rodriguez-Lazaro, Hernandez, et al., 2004), and the PCRs were run in the ABI 7700 platform (Applied Biosystems, Foster City, USA). The PCR method detected *L. monocytogenes* in all dilutions of fermented pork sausage, cooked ham and frankfurter sausage, showing similar performance ($P > 0.001$) to when purified DNA was used, indicating that the PCR system accurately detects and quantifies *L. monocytogenes* DNA in processed meat products. However, the PCR method did not detect *L. monocytogenes* in any of the raw pork meat samples. This lack of *L. monocytogenes* detection was accompanied by a lack of IAC signal, indicating that the failure to detect *L. monocytogenes* DNA was a false-negative result due to inhibition of the PCR. In a previous study of the same research laboratory (Rodriguez-Lazaro, Jofre, Aymerich, Hugas, & Pla, 2004), different pre-PCR treatments were evaluated to increase the limit of detection and quantification of *L. monocytogenes* in meat products. Twenty-five gram samples of different meat products (raw pork meat, fermented pork sausage, cooked ham and frankfurter sausage) were artificially contaminated with decreasing amounts of *L. monocytogenes* down to 1 CFU g^{-1}, and subsequently diluted (1:10) with 0.1% peptone–0.85% NaCl and homogenized for 1 min. These homogenized samples were subjected to a two-step filtration, first through a 22- to 25-μm-pore-size filter (Miracloth filter; Calbiochem) and then through a nylon membrane with an 11-μm pore size (Millipore). Then 2 ml was centrifuged for 5 min at $10{,}000 \times g$ at 4°C. The pellet was suspended in 100 μl of a suspension of 6% Chelex-100 resin (Bio-Rad, Munich, Germany) in water, incubated at 56°C for 20 min, vortexed, boiled for 8 min, vortexed again and immediately chilled on ice. Finally, the sample was centrifuged for 5 min at $14{,}000 \times g$, and 1 μl of the upper (clean) part of the solution was transferred to the mixture (Rodriguez-Lazaro, Hernandez, et al., 2004). The method allowed quantification of target down to 10^3 *L. monocytogenes* CFU/g, and detection of as few as 10^2 CFU/g in at least 50% of the replicates. It should be noted that currently there is only a very limited need for quantitative pathogen detection in pathogen testing in the meat industry (at present only *L. monocytogenes* in meat products), and also most methods still need a short enrichment period (as is detailed in most of the examples in this chapter), making quantification from the PCR reaction back to the original sample very doubtful.

O'Grady, Sedano-Balbas, Maher, Smith, and Barrya (2008) developed a real-time PCR method for the detection of *L. monocytogenes* in naturally and artificially contaminated meat samples after a 30-h-enrichment step. The target

was the *ssrA* gene encoding for tmRNA, which rescues stalled ribosomes and clears the cell of incomplete polypeptides. The detection strategy was based on fluorescence resonance energy transfer (FRET) hybridization probes using the LightCycler (Roche, Basel, Switzerland) as the real-time PCR platform. The method was fully specific, with a limit of detection of 1–10 genome equivalents. For its application in food analysis, in three independent experiments, 25 g of different meat products (hotdog, sliced ham, pate and sliced turkey) was independently added to 225 ml of half Fraser broth (Oxoid, Hampshire, United Kingdom) and homogenized in a stomacher for 2 min. For each type of meat product two samples were used: one served as a control, and the second sample was inoculated with 100 μl of a TSB-Y overnight culture of *L. monocytogenes* strain NCTC 7973 containing 10–50 CFU ml^{-1}. Both samples were incubated at 30°C for 22 h with shaking, and then 100 μl was added to 10-ml Fraser broths, respectively, and incubated at 37°C for 4 h with shaking. Finally, 1.5-ml aliquots of the secondary enrichment cultures were used for the DNA isolation using the Bacterial Genomic DNA purification Kit (Edge BioSystems, Gaithersburg, Maryland, USA). The PCR method detected *L. monocytogenes* in all artificially contaminated meat samples and did not detect any in the control samples. These results were confirmed by culturing the samples.

Future Perspective

A search of the PubMed database (www.pubmed.com) showed that at the time of writing, there had been 662 research papers published which described molecular methods (PCR-, real-time-PCR- and NASBA-based methods) for detection of the above pathogens in meat and meat products (Fig. 16.1). Considering the total effort underlying each scientific publication (researchers' salaries, materials and reagents, etc.), one may make an assumption that the total cost behind each paper is around 10, 000–20,000 euros. Thus, the overall activity which has resulted in all these methods is around 6–13 million euros. The ultimate source of research funding is the taxpayer, and the taxpayer as consumer is the prime stakeholder in every food safety research endeavour. However, the outcomes of the 6–13 million euros have not for the most part been translated into tangible benefits for the prime stakeholder. This is because very few if any of the accrued methods have been transferred from the researcher/developer's laboratory to the analyst's laboratory. Therefore, the stakeholder is not getting an adequate return for their investment.

Why is this? The typical response to this question has been that the cost of the equipment and reagents is high and it is difficult to find adequately trained personnel. However, year on year a wider choice of new platforms for real-time PCR is becoming available (from only two or three types of equipment in the late 1990 s to more than 20 available in the market currently), and there are many biotechnology companies now which offer high-quality reagents and

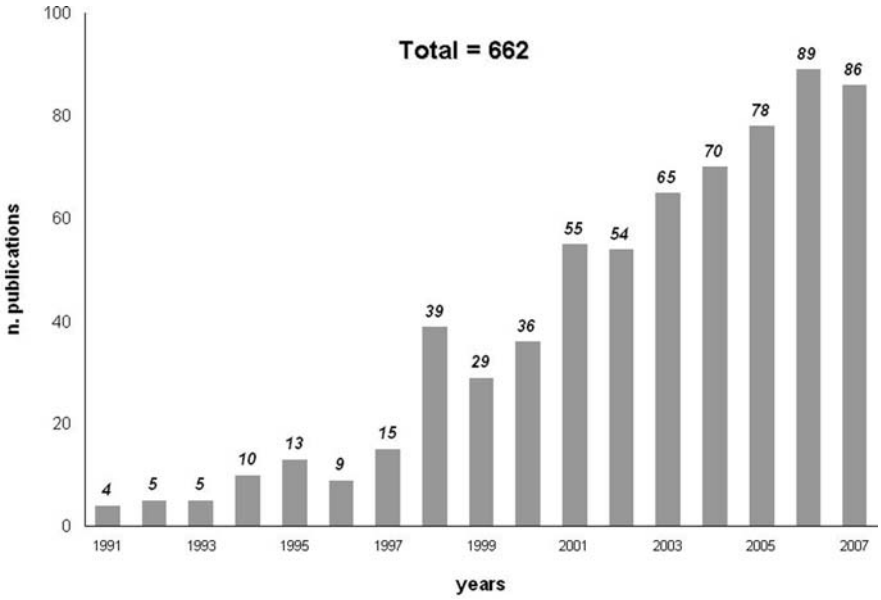

Fig. 16.1 A PubMed search using "real-time PCR", "PCR" and "NASBA" for the main meat bacterial pathogens reviewed in this chapter, sorted by year of publication

enzymes. In addition, more than 10 years have passed since the first publication in real-time PCR in 1996, and increasing numbers of trained analysts exist. So, what are the current underlying reasons hindering the adoption of these powerful methods? They are their lack of international validation in comparison with the culture-based microbiological standards, and even more importantly the food industry's lack of acceptance of them. For example, the industry is reluctant to accept PCR-positive results as unequivocal proof that a sample is contaminated with the analyte pathogen: they would insist that any such results are confirmed by conventional culture of the sample. Therefore, an advantage of the molecular-based methods, rapidity of producing results, would appear to be lost. However, this could be turned to advantage by promoting the use of PCR-based methods in screening of samples: PCR-negative samples could be cleared for consumption while positives undergo confirmation.

Ultimately, there needs to be a focused drive towards taking proven methods from the scientist's laboratory and implementing them in actual use in the analyst's laboratory. However, further developments are needed for an effective implementation of amplification techniques in food microbiology. Among the main issues that must be addressed for the effective adoption of molecular techniques by food analysis laboratories are the development of rational and easy-to-use strategies for sample treatment, and greater automation of the whole analytical process. The absolute prerequisite for successful adoption of molecular-based diagnostic methodology is international validation and

subsequent standardization (Hoorfar & Cook, 2003; Malorny, Tassios, et al., 2003; D'Agostino & Rodríguez-Lázaro, 2009). Most analysts still regard the conventional "gold standard" culture-based methods as the only accepted method. Therefore, any molecular-based method should be shown to work at least as well as the corresponding conventional method, by direct comparison of the analytical performance of each on identical food samples. There is an international standard guideline for performing this validation (Anonymous, 2003a). Standard guidelines regarding the use of PCR for the detection of foodborne pathogens have also been established (Anonymous, 2005a, 2005b, 2006a, 2006b). Finally, a determined effort to promote dialog between the researcher and the analyst is necessary, to encourage and mediate adoption of fit-for-purpose methodology. Ideally, this effort requires the establishment of a solid international infrastructure for taking promising analytical methods through development and validation and finally delivering them for use. The foundation of this scenario awaits support from international funding agencies.

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Chapter 17
Detecting and Tracking Emerging Pathogenic and Spoilage Bacteria from Farm to Fork

Geraldine Duffy

Introduction

There is a direct relationship between the microbiological profile of food and its safety and quality. A knowledge of the typical microbiological profile (i.e. microbial species) associated with a particular meat or meat product as well as information on the microbial load is important in terms of accurately predicting both its safety and its shelf life.

Emergent Pathogens

Zoonotic pathogens can be transmitted from animals to humans through the meat chain and can cause a significant burden of illness. *Campylobacter*, *Salmonella, Listeria monocytogenes* and verocytotoxigenic *E. coli* (VTEC) group, which includes *E. coli* O157:H7, are significant causes of bacterial gastrointestinal illnesses in humans (EFSA, 2007). In recent years, a combination of factors have led to the emergence of additional zoonotic micro-organisms in the meat chain. A continuing shift away from small farming units to very large-scale intensive farming operations has given enormous potential for widespread dissemination of zoonotic pathogens among large numbers of animals and the wider environment, giving emerging organisms access to new niches and host environments. Intensive farming has also created the need for large-scale use of anti-microbials including antibiotics in animal production to control and prevent spread of infection among large numbers of cohort animals. A knock-on effect from this has been an exponential increase in anti-microbial resistance and often multi-antibiotic resistance in bacteria isolated from meat (McDermott et al., 2002). Multi-antibiotic-resistant *Salmonella typhimurium* DT104 is now one of the most commonly isolated *Salmonella* species from pig meat in Europe (EFSA, 2007).

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Microbes also possess a remarkable ability to develop new resistance mechanisms and pathogenic vigour to survive hostile environments and propagate. In any hostile environ, subpopulations of micro-organisms can potentially develop resistance to biological, chemical or physical stresses and over time an adaptation can be transferred to progeny. This adapted population can potentially become dominant over the wild-type organism leading to the emergence of bacterial populations with enhanced pathogenic vigour and survival mechanisms (Levy, 1998; Hall, 2004). A recent example is the continuing emergence of virulent verocytotoxigenic *E. coli*, and *E. coli* O26, O145, O103, O111 and O91 in addition to *E. coli* O157:H7 are now classed as being of public health concern (EFSA, 2007).

Emergent Spoilage Micro-organisms

The shelf life of meat or meat products can be predicted by estimating the number of micro-organisms present at a particular time point in the chain and predicting the increase in the micro-flora which will occur during distribution and storage under a defined set of environmental conditions such as temperature, pH and a_w. Spoilage can be defined as the time when the micro-flora reaches a critical level usually at around $\log_{10}$ 7–8 colony-forming units (CFU) g^{-1}, at which time the flora has induced sufficient organoleptic changes to render the meat unacceptable to the consumer. Spoilage occurs because as micro-organisms proliferate on the meat they metabolise the proteins, fat, carbohydrates, etc. in the meat into smaller breakdown products giving rise to the off odours and colours typically associated with spoiled meat. While it would be unusual to routinely identify specific spoilage organisms on meat, there are instances where this level of information is helpful in predicting spoilage. The principal genera of bacteria are present on meat that include *Pseudomonas* spp., *Acinetobacter* spp., *Aeromonas* spp., *Brochothrix thermosphacta*, members of the lactic acid bacteria (LAB) such as *Lactobacillus* and *Leuconostoc*, as well as many members of the Enterobacteriaceae including *Enterobacter* and *Serratia* spp. (Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Hinton, Cason, & Ingram, 2004; Borch, Kant-Muermans, & Blixt, 1996; Huis in't Veld, 1996; Gustavsson & Borch, 1993). The environmental conditions during processing and storage influence which of the diverse micro-flora will proliferate. Under aerobic chilled storage conditions, certain species of the genus *Pseudomonas* contribute significantly to spoilage. This is due to the organisms' ability to utilise amino acids and grow well at refrigeration temperatures. Although it is a facultative anaerobe, under anaerobic conditions, the bacterium *B. thermosphacta* can become a dominant member of the spoilage flora of meat products, producing lactic acid and ethanol as by-products of glucose utilisation (Pin, Garcia de Fernando, & Ordonez, 2002). The use of modified atmosphere packaging (MAP) is now a very common method of preservation. Gas mixtures containing variable O_2

and CO_2 concentrations are used to inhibit the growth of different spoilage-related bacteria. Under modified atmosphere packed storage conditions, lactic acid bacteria are prolific spoilers (Chenoll, Macian, Elizaquivel, & Aznar, 2007).

In recent years, there has been an increased demand for fresh meat and meat products which have a longer shelf life, yet a shift away from the use of traditional preservation methods which are deemed to impact adversely on the quality or health of the meat such as freezing, salt, sodium nitrite, canning. As these agents and processes were all excellent preservatives it creates a major challenge to extend life in their absence. Equally a global export market requires fresh meat primals to have a longer shelf life. This has lead to increased use of packing technologies and multiple-hurdle technologies to extend the shelf life of fresh meats. However, changes in the storage environment can also lead to changes in the natural micro-flora balance of the food and may give an emerging pathogen or spoilage micro-organism a competitive advantage to survive and propagate. For example, an emergent problem over the last 15–20 years associated with storage of meat under chilled conditions in vacuum packs is the sporadic contamination and proliferation of psychrophilic *Clostridia* species (*C. estertheticum* and *C. gasigenes*) on meat stored under these conditions (Collins, Rodrigues, Dainty, Edwards, & Roberts, 1992; Broda, Saul, Lawson, Bell, & Musgrave, 2000). The proliferation of these *Clostrida* is highly problematic leading to extensive gas production and so-called blown packs causing major economic losses when it effects, for example, large vacuum-packed meat primal cuts. The most probable sources of these *Clostrida* in the meat plant environments are the animal hide, soil particles and faecal material (Boerema, Broda, & Bell, 2003).

Recent investigations on New Zealand chilled vacuum-packaged meats have shown that Enterobacteriaceae may also have a role in blown pack spoilage (Brightwell, Clemens, Urlich, & Boerema, 2007). This study showed moderate to high numbers of Enterobacteracea in the spoilage flora, but no *C. estertheticum* and *C. gasigenes* that are usually associated with blown pack spoilage. The study showed that pyschrotolerant Enterobacteriaceae including *Enterobacter*, *Serratia*, *Hafnia* and *Rahnella* produced gas in a lamb homogenate model under anaerobic conditions and gas production was also confirmed in vacuum-packaged lamb shoulders stored at 4°C for 21 days after being inoculated with individual representative Enterobacteriaceae isolates.

Detecting Micro-organisms on Meat

Food hygiene regulators and industry set microbiological guidelines for key pathogens to assure meat safety. Quantitative guidelines are set for total viable counts on carcasses, processed raw meats and ready-to-eat meats, etc. to ensure that under a particular set of defined environmental parameters the meat will have the required and predicted shelf life.

Total Bacterial Counts

The most common approach to quantify micro-organisms (total viable count) for the purposes of predicting shelf life and spoilage is culture based and dependent on the growth of a microbial population to form visible morphologically distinct colonies on an agar plate. The gold standard culture-based method is the aerobic standard plate count (SPC) and all microbial criteria are based on this approach. The Association of Official Analytical Chemists (AOAC) Official Method 966.23 (AOAC, 1990) and the International Organization for Standards (ISO) (No. 4833:2003) (ISO, 2003) have standardised the test protocol. All alternative methods must generally be correlated or validated against these methods. Some of the alternative and generally more rapid methods which have been developed to estimate total microbial numbers include direct epifuorescent filtration methods (DEFT) (Liberski, 1990; Duffy, Sheridan, McDowell, Blair, & Harrington, 1991; Sierra, Sheridan, & McGuire, 1997; Duffy & Sheridan, 1998), ATP bioluminescence methods (Siragusa, Dorsa, Cutter, Perino, & Koohmaraie, 1996; Ellerbroek & Lox, 2004), electrical methods (Bollinger, Casella, & Teuber, 1994), spectroscopic-based methods (Ellis, Broadhurst, Kell, Rowland, & Goodacre, 2002; Ellis, Broadhurst, & Goodacre, 2004) and electronic noses (Blixt & Borch, 1999; Du et al., 2001). All these reported methods have varying levels of correlation with the standard culture method.

A key contributor to the spoilage of fresh meat is a breakdown in the chill chain during distribution. Systems or methods which can continuously monitor temperature during chilled storage and distribution would thus be very beneficial in accurately predicting shelf life. Time temperature integrators (defined as small, inexpensive devices that can be incorporated into a meat package and give a recorded history or show a visible change dependent on the time and temperature history of the stored meat) are now receiving considerable research and development attention and have great potential to better predict and monitor the microbiological status of meat in real time (Moore & Sheldon, 2003).

Detecting Specific Pathogens or Spoilage Micro-organisms

There are many challenges in the detection of specific pathogens or spoilage micro-organisms on meat which include the fact that they are generally present in very low numbers in the meat (often < 100 cfu g^{-1}) and sometimes in the midst of up to one million other micro-organisms and they may be on the surface or, for example, imbedded in a comminuted meat product. The micro-organisms on the meat are often in an injured/stressed condition and may be in a viable but nonculturable state. Traditional methods for the detection of specific bacteria rely on culturing of the organisms on agar plates (1–2 days) and may also require an initial liquid enrichment step (1–2 days) to increase the numbers of the target organism to detectable levels. The suspect colony

on the agar plate can then be identified by morphological, immunological or biochemical means (De Boer & Beumer, 1999). These methods are thus very time consuming taking 5–7 days to detect specific pathogenic micro-organisms.

Alternative and more rapid approaches to detect specific micro-organisms have been developed based on the use of either immunological or molecular methods to detect the micro-organism following a period of liquid enrichment.

Immunological methods are based on a reaction between a specific antigen on the target micro-organism and a complementary antibody. Immunoassays incorporating enzyme-labelled antibodies are the most commonly used type of immunoassay for the detection of specific pathogens. There are many possible enzyme-linked immunoabsorbent assay (ELISA) formats but the "sandwich" assay is most often used in commercially available tests. An antibody bound to a solid surface which may be a micro-titre plate, plastic strip, dip stick, capillary migration system, etc. acts to capture the antigen (on the target micro-organism). The second antibody, conjugated with an enzyme, binds to the captured antigen and finally an appropriate substrate is added to give a visible colour change or a measurable change in fluorescence which is readable by a fluorescent reader. ELISA detection methods are amenable to automation and can handle high sample throughputs and are useful for screening large number of samples. Commercial ELISA methods are available for a range of meat pathogens including *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7. They generally require 10^5–10^6 cfu per ml of target cell in the enriched meat sample and require a 24–48 h enrichment period prior to applying the test.

Major advances in the level of genomic information available for food-borne pathogens are now being exploited to develop molecular methods to detect micro-organisms in foods. These methods are based on the detection of a specific piece of genetic material (a specific sequence of nucleic acids, i.e. DNA or RNA) which is unique to the target organism and as such they are highly specific.

Nucleic acid methods which include an amplification step for the target DNA/RNA are now routinely employed in molecular biology. These methods increase the target nucleic acid material by up to a million fold and are particularly important in the arena of food microbiology where one of the major hurdles is the recovery and detection of very low numbers of a particular pathogen. The most popular method of amplification is the polymerase chain reaction (PCR) technique. In this technique, the DNA is extracted from the organism and the double strands are denatured into single-stranded DNA. Short-sequence DNA primers are annealed to the complementary DNA target in the organism. The primers are then extended across the target sequence using a heat-stable DNA polymerase (usually *Taq* polymerase, a thermostable and thermoactive enzyme from *Thermus aquaticus*) in the presence of free deoxynucleoside triphosphates (dNTPs) resulting in a double replication of the starting target material. Multiple repeats of the denaturation, annealing and extension steps result in an exponential increase in the levels of the initial target DNA, thus

greatly increasing the sensitivity of the method (Entis et al., 2001). In conventional PCR the amplified product is detected by staining with ethidium bromide on an electrophoresis gel and this type of PCR has been successfully applied to the detection of a range of pathogens from meat and meat products including *Salmonella* (Chiu, Chen, Hwang, & Tsen, 2005; Jin et al., 2004), *Listeria monoctyogenes* (Jung, Frank, Brackett, & Chen, 2003), *Campylobacter* (Mateo, Carcamo, Urquijo, Perales, & Fernandez-Astorga, 2005) and *E. coli* O157:H7 (Fitzmaurice et al., 2004). PCR can also be used to detect specific spoilage micro-organisms on meat including psychrophilic *Clostridium* spp (Broda, Boerema, & Bell, 2003; Boerema, Broda, & Bell, 2002) and spoilage causing lactic acid bacteria (Yost & Nattress, 2000) and *Leuconostoc* in meat processing plants (Goto et al., 2004; Lee, Park, & Kim, 2000).

Real-time PCR is now increasingly replacing conventional PCR as a rapid, sensitive and specific molecular diagnostic technique (Bellin, Pulz, Matussek, Hempen, & Gunzer, 2001). Real-time PCR allows continuous monitoring of amplification through the use of fluorescent double-stranded (ds) DNA intercalating dyes or sequence-specific probes (Wittner, Herrmann, Moss, & Rasmussen, 1997) and offers many advantages over traditional PCR methods being much quicker to perform and the use of a closed system for amplification and detection minimises the potential for amplicon carryover contamination (Bankowski & Anderson, 2004).

Real-time PCR technology has been used to detect a range of meat-borne pathogenic micro-organisms including *Salmonella* (Catarame, O'Hanlon, Blair, McDowell, & Duffy, 2006; Ellingson, Anderson, Carlson, & Sharma, 2004), *Listeria monocytogenes* (Wang, Jothikumar, & Griffiths, 2004; Lunge, Miller, Livak, & Batt, 2002), *E. coli* O157 (Sharma, 2002; O'Hanlon et al., 2004) and emergent VTEC such as *E. coli* O26, O111, O145 and O103 (O'Hanlon et al., 2004; Perelle, Dilasser, Grout, & Fach, 2004).

Tracking Micro-organisms

In the event of an outbreak of food-borne microbial infection it is essential to be able to track and categorically identify the source of contamination so that measures can be put in place to ensure no further consumer exposure occurs. Equally it is important to be able to categorically track the route(s) by which pathogenic bacteria potentially pass from the animal through the meat chain to the consumer so that routes of transmission and sources of cross-contamination are identified and measures and resources then targeted at the high-risk stages of the chain. Equally when sporadic cases of spoilage micro-organisms such as the psychrophilic *Clostrida* sp., or lactic acid bacteria, etc. occur it is essential that the source of contamination be categorically identified and decontamination measures then focused on the key areas.

Tools for Tracking Bacteria

There are a number of approaches which can be used to characterise and thus compare micro-organisms at a strain level referred to as typing and sub-typing methods. Typing methods can be classified as phenotypic (detecting a characteristic(s) expressed by the bacteria) or genotypic (directly examining the bacterial genetic content). Phenotypic methods including phage typing, serotyping and biotyping and antibiograms are widely used but genotypic techniques are increasingly used as they are much more discriminatory and yield substantially more information about the isolate.

The most discriminatory approach to categorically relate bacteria is to obtain a genetic profile/fingerprint of the isolate and to then compare the genetic fingerprints for each isolated strain. There are a number of genetic fingerprinting approaches and the basis of these methods and their application to spoilage or pathogenic micro-organisms from meat are described below.

Pulsed Field Gel Electrophoresis

One of the most commonly applied genetic fingerprinting approaches is pulsed field gel electrophoresis (PFGE) (Méndez-Álvarez, Pavòn, Esteve, Guerrero, & Gaju, 1995). PFGE is a method for separating large DNA molecules in a unique manner by employing restriction enzymes (endonucleases) to make a limited number of cuts in the bacterial chromosome. This provides a unique chromosomal restriction pattern or "fingerprint" for each individual bacterial isolate. When a fingerprint is generated, computer software (bioinformatics) is used to compare the profiles with that of other bacterial isolates and establish how closely they are related. As PFGE methodology, software, etc. are highly standardised internationally it has facilitated the development of national and international databases of generated PFGE profiles. The largest of these is called "PulseNet" (www.cdc.gov/pulsenet/) which is a North American database coordinated by the Centres for Disease Control and Prevention (CDC) which contains thousands of PFGE profiles for a range of food pathogens. Detailed procedures for generating PFGE profiles for *Salmonella*, *Campylobacter, Listeria monocytogenes* and *E. coli* O157 can be downloaded from the pulsejet website. In Europe, there are a number of smaller databases generally administered by national pathogen reference laboratories, and the European Centre for Disease Prevention and Control (ECDC) plan to establish an EU-wide "Pulsenet Europe" database (Gerner-Smidt & Scheutz, 2006).

Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis investigates certain types of sequence polymorphisms, so-called point mutations which can be base exchanges, base deletions or insertions. The

basic mechanism of RFLP analysis relies on the ability of restriction enzymes, the "endonucleases", to cut double-stranded DNA according to a certain succession of bases in a process called digestion. RFLP can be performed by digestion of DNA samples followed by analysis using standard gel-transfer hybridisation procedures. Another method is the restriction digestion of a PCR-amplified DNA segment that contains a variably present restriction site. The technique thus requires some knowledge of the DNA sequence flanking that restriction site (Dietrich, Weber, Nickerson, & Kwok, 1999). RFLP has been applied to differentiate reference and meat strains of psychrophilic and psychrotrophic *Clostridia* (Broda, Musgrave, & Bell, 2000) and has also been used to characterise lactic acid bacteria associated with vacuum-packed cooked meat product spoilage (Chenoll et al., 2007)

Multi-locus Sequence Typing (MLST)

Multi-locus sequence typing (MLST) exploits the unique nucleotide sequences in micro-organisms by amplifying and sequencing segments of a number of housekeeping genes. Between isolates there will be so-called point mutations which can be base (A, T, G, C) exchanges, base deletions or insertions and the sequences from different isolates can be compared to establish relatedness. This tool has been applied to a wide range of food-borne pathogenic bacteria (Urwin & Maiden, 2003), and there is a database (http://www.mlst.net) of profiles which also contains specific protocols. This technique has been shown to be particularly useful for sub-typing *Campylobacter* spp. (Miller et al., 2005).

Multi-locus Variance Analysis(MLVA)

Most bacterial genomes contain tandem duplications of short DNA sequences, termed "variable-number tandem repeats" (VNTR). Repeat unit sizes and repeat sequences can vary when multiple loci (site on a chromosome where a gene is located) are examined in a number of different isolates of an individual microbial species. It has been documented on many occasions that the number of repeat units per locus is a strain-defining parameter. Consequently, there is isolate specificity in the number of repeats per locus, when different strains of a given bacterial species are compared. A sub-typing method targeting these repeats, multiple-locus VNTR analysis (MLVA), has emerged as a powerful tool for characterisation of bacterial pathogens (Van Belkum, 2007). MLVA has been used effectively for tracing *Salmonella* and VTEC and is a modern, timely and versatile bacterial typing methodology (Hyytia-Trees, Smole, Fields, Swaminathan, & Ribot, 2006; Torpdahl, Sørensen, Lindstedt, & Nielsen, 2007). In a recent outbreak related to *E. coli* 0103 in fermented sausage in Norway, MLVA gave more discriminatory results than PFGE. (Schimmer et al., 2008).

Micro-arrays

The next generation of typing and tracking technology is likely to be micro-arrays. These can consist of a range of specific gene sequences (nucleotides) immobilised on surfaces such as micro-titre plates, micro-beads, micro-wells or eppendorfs tubes (Venkatasubbarao, 2004). The underlying principle of micro-array analysis is the hybridization of nucleic acid strands on the micro-array with the corresponding complimentary sequences in the target micro-organism. There is the capacity to put a wide range of specific genes, such as virulence genes, into this format or whole genome information. Micro-arrays can be used to detect and characterise bacteria but also to investigate gene expression. The analysis of the information generated where tens of thousands of nucleotides are involved is tremendous and requires highly specialist bioinformatics. The cost of micro-arrays currently prohibits their widespread use for routine purposes, but as they are more widely used, costs will continue to drop and their use will correspondingly increase.

Conclusion

For the meat industry, detection and tracking of known and emerging micro-organisms in the meat chain can give scientific evidence about where pathogenic or spoilage micro-organisms are entering the meat chain. It can thus give clear guidance on where cross-contamination may be occurring, and whether particular strains are endemic in a factory environment, allowing risk-based management decisions on where controls should be directed.

There is also huge potential value from genomically comparing bacteria from human infection with those isolated from meat or animal sources as it will establish whether there are particular animal host reservoirs for strains of the pathogen which are more virulent for humans. As molecular tools advance the concept of molecular risk assessment will likely become routine with all strains recovered from food assessed for virulence potential based on their gene profile and relatedness to human clinical isolates and risk management decisions made based on the outcome. This data will enable focusing of resources most effectively on high-risk vectors and vehicles of contamination to reduce the overall public health risk posed by that pathogen.

As the agri-food sector and indeed society continue to evolve, micro-organisms will continue to evolve in parallel and this will no doubt lead to the emergence of new food safety and or spoilage issues, including newly recognised or adapted micro-organisms and new routes and vectors of transmission. It is thus essential that that the latest technological developments for detection, tracking, surveillance and reporting systems be taken on board and integrated nationally and internationally. This will allow emergent threats to be identified in a timely manner. Equally greater awareness and scientific research on the impact of novel meat

production and processing methods on the total meat micro-flora will help ascertain where certain practices are encouraging bacterial adaptation and the emergence of new microbial threats and meat safety issues.

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Chapter 18
Molecular Analysis of Pathogenic Bacteria and Their Toxins

Catherine M. Logue and Lisa K. Nolan

Introduction

Use of molecular methods for investigation of foodborne pathogens and illness has become much more commonplace over the last decade or so. Application of these methods has significantly expanded fields of inquiry related to food safety. Molecular methods have been used to facilitate isolation and detection of pathogens and to enhance subtype analysis of strains in an effort to link or determine relationships between strains and hosts and to sources of contamination.

Although many molecular methods rely on the presence of a pure population of cells for analysis, a considerable number of protocols exploit molecular-based techniques for isolation of target pathogens from mixed populations in foods. Such isolation protocols increase the population of the cells of interest to levels that can subsequently be analyzed by a molecular method. As traditional methods are labor and time intensive, and in the case of rapid kits often expensive, such molecular methods can provide an attractive alternative for pathogen detection.

With increased globalization of the food/meat supply is likely to come an increased reliance on molecular methods for investigations related to food safety. Since the source of a food may be thousands of miles and continents away from where it is consumed, the ability to analyze, detect, and link a pathogen with host and environment becomes complicated. Molecular analysis offers a useful means to epidemiologically trace back a pathogen to source, while also providing useful information about the genetic constitution of the pathogen and/or its abilities to respond to the host or its environment. Thus, judicious use of molecular analysis tools and methods can assist investigators in wise application of limited resources to the benefit of public health and food safety.

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Here, we will focus on molecular methods associated with pathogens from meats/foods that have been identified to species level. In such settings, molecular methods allow researchers the opportunity to ascertain what genes, proteins, and mechanisms are available to enhance the persistence of organisms in foods and the environment; determine why some strains are more commonly isolated in certain foods; and elucidate the basis of their pathogenicity (Fratamico & Bales, 2005).

Choice of an appropriate molecular analysis method is based on the type of information required by the investigation. In some cases, the purpose of the investigation is to epidemiologically link a pathogen to its source, where a similar pathogen has been found. However, if the question is more complex, then additional analysis needs to be considered (Foxman, Zhang, Koopman, Manning, & Marrs, 2005). In all cases it must be understood what the outcomes of each method are and how its results can be applied. Where molecular methods are considered for subtyping, the results of the analysis involve characterization of the pathogen below the species or subspecies level (Hyytia-Trees, Cooper, Ribot, & Gerner-Smidt, 2007; Struelens, 1996). For further insight into subtyping techniques, the reader is advised to review some recent overviews of molecular subtyping and its application in epidemiological studies (Foxman et al., 2005; Hyytia-Trees et al., 2007; Van Belkum et al., 2007; Wiedmann, 2002).

Non-nucleic Acid Typing Methods

Although this chapter focuses on nucleic acid typing methods, use of one phenotypic method is included here, as it is a precursor to a nucleic acid typing method discussed later.

Multi-locus Enzyme Electrophoresis—MLEE

MLEE is a typing technique that produces a characteristic electrophoretic profile based on genetic variation in conserved cellular enzymes. The principle behind this technique is that genes that encode housekeeping enzymes tend to be highly conserved (Smith, Feil, & Smith, 2000), including within a genus (Selander et al., 1986). MLEE detects differences in genes that encode these housekeeping enzymes. Such changes are detectable since changes at the amino acid level will result in changes in net electrostatic charges of the protein, which, in turn, will affect enzyme migration during electrophoresis. These differences are then used to generate profiles of the strains under study.

In MLEE, bacteria under investigation are lysed in such a way so as to avoid protein denaturation, and cytoplasmic enzymes are separated out by electrophoresis. Enzyme presence is visualized with an enzyme substrate and dye. The distance of enzyme migration is measured and assigned a number

corresponding to an allele. Enzymes that migrate the same distance are more likely to be the same alleles than those with different migration patterns. However, one must use caution in interpreting the results of MLEE, as it is possible to have different enzymes that migrate the same distance. Although MLEE is a relatively common method for subtyping bacteria, it has been supplanted, in large measure, by methods that target DNA for sequencing, such as multi-locus sequence typing (MLST see below), which has greater reproducibility than MLEE (Maiden et al., 1998).

Nucleic Acid Typing Methods

Plasmid Analysis

Plasmids are extrachromosomal, usually circular, forms of DNA found in many bacteria. Plasmids are said to encode accessory functions, as opposed to chromosomes which encode essential functions (Levin & Bergstrom, 2000). Plasmid function varies in different bacteria but roles in virulence, selective advantage, and antimicrobial resistance are well known (Bergstrom, Lipstitch, & Levin, 2000; Carattoli, Villa, Pezzella, Bordi, & Visca, 2001; Carattoli et al., 2006; Foley & Lynne, 2007; Frost, Leplae, Summers, & Toussaint, 2005; Johnson, Siek, Johnson, & Nolan, 2005; Johnson, Siek, Johsnon, & Nolan, 2006; Johnson, Johnson, & Nolan, 2006; Johnson, Wannemuehler, Scaccianoce, Johnson, & Nolan, 2006; Levin & Bergstrom, 2000; Levy, Fitzgerald, & Macone, 1976). The plasmid content of bacteria can be exploited in typing and ascertaining relationships among strains. In plasmid profiling, plasmid DNA can be isolated from the strain of interest, and the size of the strain's plasmids is estimated using agarose gel electrophoresis. However, estimation of plasmid size can be problematic, especially when the DNA is not digested with restriction enzymes prior to analysis. Plasmid DNA may occur in different states of coiling, which will impact migration through agarose during electrophoresis. Complex banding patterns due to simultaneous occurrence of different states of the same plasmid can negatively impact interpretation of the results. Also of note is the possibility that two strains of interest might harbor different plasmids of similar size. In an unrestricted state, the plasmids of such strains might appear the same following electrophoresis, while being considerably different. Despite its drawbacks, plasmid analysis does have value in certain epidemiological investigations (Aktas, Day, Kayacan, Diren, & Threlfall, 2007; Miles, McLaughlin, & Brown, 2006; Petersen, Christensen, Kuhnert, Bisgaard, & Olsen, 2006).

To overcome these limitations, plasmid DNA can be subjected to digestion with restriction endonucleases prior to electrophoresis to generate a "fingerprint" (see Fig. 18.1). Despite the fact that this method provides a much more accurate picture of a strain's plasmid content, its use is somewhat limited in epidemiological investigations. It only works for strains possessing

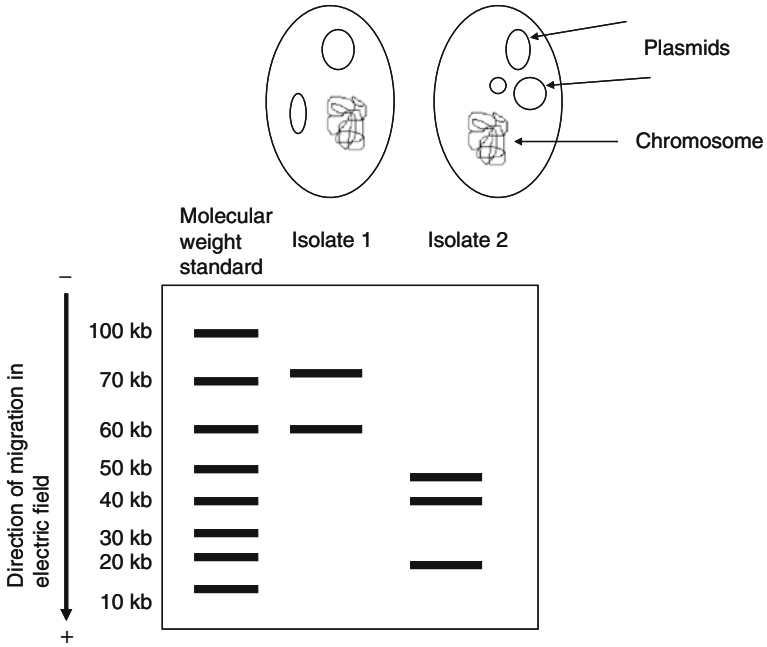


Fig. 18.1 Plasmid analysis

plasmids, and plasmids themselves may be unstable, making profiling difficult. Plasmid profiling has been used with a range of bacteria including *Salmonella* (Aktas et al., 2007; Fakhr, Sherwood, Thorsness, & Logue, 2006; Holmberg, Wachsmuth, Hickman-Brenner, & Cohen, 1984; Horby et al., 2003; Millemann, Lesage, Chaslus-Danica, & Lafont, 1995; Olsvik et al., 1985; Wachsmuth et al., 1991), *Escherichia coli* (Johnson, Wannemuehler, et al., 2007; Li, Sherwood, & Logue, 2007; Miles et al., 2006; Petersen et al., 2006), *E. coli* O157:H7 (Pradel, Bertin, Martin, & Liverelli, 2008; Radu, Ling, Rusil, Karim, & Nishibuchi, 2001), *Listeria* (Harvey & Gilmour, 2001; Kolstad, Caugant, & Rorvik, 1992; Vaz-Veiho, Duarte, McLaughlin, & Gibbs, 2001), and *Campylobacter* (Aquino et al., 2002; Fayos, Owen, Hernandez, Jones, & Lastovica, 1993; Lekowska-Kochaniak, Rozynek, & Popowski, 1996; Mazi et al., 2008).

Also, replicon typing of plasmids may have some value in investigation of relatedness among foodborne pathogens. Recently, simplified methods, using multiplex PCR to identify common replicon types occurring in the Enterobacteriaceae, have been described (Carattoli et al. 2005; Johnson, Wannemuehler, et al., 2007). Use of these methods has been applied to plasmids of isolates recovered from poultry, poultry meat, other animals, and foods of animal origin (Carattoli et al. 2005; Johnson, Wannemuehler, et al., 2007).

Amplification-Based Methods

Polymerase Chain Reaction

PCR has revolutionized molecular studies. The principle of the technique is that a thermostable DNA polymerase can be used to amplify a specific region of DNA to detectable levels based on template DNA found in the microorganism of interest. In conventional PCR methodology, amplicons are then separated out by gel electrophoresis, the DNA stained, and the size of the DNA bands determined by comparison to standard DNA. Use of such amplification techniques has expanded considerably over recent years and is used in identification of bacteria, detection of pathogens in foods and other substrates, detection of genes associated with pathogenesis, virulence, or toxins, and detection of viruses or fungi (Auvray et al., 2007; Baert, Uttendale, & Debevere, 2008; Kim et al., 2007; Niessen, 2008).

Newer PCR techniques are emerging, making this technique ever more useful. For example, PCRs are commonly configured to amplify multiple gene targets simultaneously. To work well, multiplex protocols use primer sets which ensure that amplification products are at least 30–50 bp apart, allowing adequate resolution following gel electrophoresis. Multiplex PCRs have been used in many ways including simultaneous detection more than one pathogen in a mixed sample or matrix, e.g., detection of multiple pathogens such as *Salmonella*, *Shigella*, *Listeria* in artificially inoculated foods (Alarcon, Garcia-Canas, Cifuentes, Gonzalez, & Aznar, 2004; Kim et al., 2005, 2007) and detection of multiple virulence or resistance genes in *E. coli* and *Salmonella* (Johnson, Wannemuehler, et al., 2007; Li et al., 2006; Nde & Logue, 2008; Skyberg et al., 2003; Skyberg, Logue, & Nolan, 2006). Martinez et al. (2006) used multiplex PCR for the simultaneous detection of three genes associated with the production of cytolethal distending toxin (CDT) in *Campylobacter jejuni* and found the prevalence of the genes to be 98% in human and animal isolates recovered from a wide European geographic origin. In a similar study, Asakura et al. (2008) developed a multiplex for *cdt* genes in *C. jejuni* and *C. coli*, with a minimum detection limit of 10–100 colony-forming units (CFU).

When PCR is used in the detection of target genes from food matrices or mixed culture, the quality of the DNA that will serve as template for amplification is of concern. Cultures and food matrices may contain substances which inhibit PCR (Fakhr, McEvoy, Sherwood, & Logue, 2006); these include products such as carbohydrates, fats, polysaccharides, and proteins. Commercial PCR clean-up kits have been developed to reduce this effect. Efficiency or utility of PCR may be enhanced using different enzymes and labeling agents. Speciality *Taq* polymerases may be used to increase the efficiency of the PCR (Fratamico & Bales, 2005), and the use of fluorescent-labeled primers has lowered detection limits. For example, Chen et al. (1998) used fluorescent-labeled primers for detection of shiga toxin-producing *E. coli* (STEC) from

foods. Primers were designed to detect the presence of the shiga toxin genes *stx1*, *stx2*, and *stxe*. Detection limits of the method were 1–5 CFU per PCR mixture (pure culture) and 3 CFU per 25 g of food. Fanning et al. (1995) used a similar technique to develop a color amplified PCR system for the detection of the heat-stable toxin gene (ST) in enterotoxigenic *E. coli* (ETEC). The method was found to have a lower detection limit of 10 fg of purified DNA and was capable of detecting 270 CFU of an ETEC strain possessing the ST gene.

In a recent review, Niessen (2008) described the application of PCR for the diagnosis and quantification of mycotoxin-producing fungi in foods and other commodities, while Zhang et al. (2008) reported a modification of PCR using an immuno-PCR assay for the detection of shiga toxin 2 (*stx2*) in culture. This modification had a significant impact on sensitivity allowing detection at a level of 10 pg/ml, as compared with commercial enzyme immunoassays which have a limit of detection of 1 ng/ml. Ge, Zhao, Hall, and Meng (2002) reported using a combination of PCR followed by enzyme-linked immunosorbent assay (ELISA) for the detection of STEC in food. Detection limits of the assay were 0.1–10 CFU, depending on the strain type. Another modification of PCR combined restriction fragment length polymorphism (RFLP) with PCR (Atanassova, Meindl, & Ring, 2001) for the detection of *Staphylococcus aureus* and staphylococcal enterotoxins in pork and pork products. This method was found to be more sensitive than standard culture techniques with a detection rate of 28.6–34.8% compared to 11.1% by standard culture. Other modifications include restriction site-specific PCR (RSS-PCR) (Kimura, Mandrell, Galland, Hyatt, & Riley, 2000) and the ramification assay (RAM) for detection of *E. coli* O157:H7 and STEC (Li et al., 2005).

Nested PCR

Nested PCR uses a primary set of primers to amplify a specific region of DNA, then a secondary set of primers is used to amplify a product which lies (i.e., nested) within the initial product. Nested PCR is designed to increase the accuracy of the PCR. That is, this dual amplification strategy ensures that if the wrong target is amplified in the primary reaction, the odds of a second amplification are low, as the second pair of primers are smaller and designed to amplify only within a region of the first PCR product. The odds of amplifying such a product in an error sequence are low. Thus the accuracy of the results is likely increased over standard methods. Nested PCR has been used in the detection of *Shigella* in food (Lindqvist, 1999; Warren, Parish, & Schneider, 2006) and for the detection of toxin-associated genes in some pathogens. Miwa, Nishina, Kubo, and Fujikura (1996) described the use of nested PCR for the detection of enterotoxigenic *Clostridium perfringens* in animal feces and meat using a pair of nested primers homologous to the *C. perfringens* enterotoxin gene (CPE). The sensitivity of the test is about 10^3-fold greater than standard PCR.

Real-Time PCR

Real-time PCR is a modification of conventional PCR methodology that eliminates the need for gel electrophoresis, thus providing results more quickly. A recent review by Mackay (2004) provides good information about the details of the technology. Whereas its underlying principle is based on PCR, a fluorescent tag is added to the primers so that the amplicons can be detected on a real-time basis by monitoring fluorescence. Thus, a detector in the thermocycler detects amplified product as it is produced. Real-time PCR protocols can be designed to detect multiple products simultaneously—typically four or five fluorescent tags can be detected together without the need for separation of product sizes as with conventional multiplex PCR products being run on a gel. Distinct advantages of real-time PCR include easy resolution of product, a faster run time, as there is no post PCR gel analysis, and increased sensitivity. The latter is greater than conventional PCR, as real-time PCR technology relies on the detection of a signal and its quantification with the release of light being in proportion to amplified product formed. Dyes such as HEX, FAM, ROX, and SYBR Green are some of the common fluorescent dyes which can be used simultaneously in a multiplex real-time PCR protocol (Huang, Hu, & Li, 2007; Nde, Fakhr, Doetkott, & Logue, 2008; Wang, Li, & Mustaphai, 2007). Multiple commercial thermocyclers for use in performing real-time PCR are available. Despite the practical advantages of this method, the expense of the requisite thermocyclers is high which may curtail use of real-time PCR in some instances. Potential applications of this method include detection of genes or strains in a range of media (Bohaychuk et al., 2007; Fakhr, McEvoy, et al., 2006; O'Grady, Sedano-Balbas, Maher, Smith, & Barry, 2008; Rodriguez-Lazaro, Jofre, Mymerich, Hugas, & Pia, 2004; Wang et al., 2007), detection of bioterrorism agents (Fykse, Langseth, Olsen, Skogan, & Blatny, 2008), mycotoxins (Bluhm, Cousin, & Woloshuk, 2004; Halstensen, Nordby, Eduard, & Klemsdal, 2006; Morello et al., 2007), and types of diarrheagenic *E. coli* (Vidal et al., 2005). Horsmon et al. (2006) developed real-time fluorogenic PCR for the detection of *entA*, the gene encoding the staphylococcal enterotoxin A (SEA). The method was capable of detecting SEA with a limit of detection of 1–13 gene copies. In a modification of real-time PCR, Fykse, Skogan, Davies, Olsen, and Blatny (2007) reported the use of a molecular beacon real-time nucleic acid sequence-based amplification for *Vibrio cholerae* by detection of the cholera toxin gene (*ctxA*) and the genes *tcpA*, *toxR*, *hlyA*, and *groEL*. The method was able to detect the organism at 50 CFU/ml and was capable of differentiation of toxigenic from non-toxigenic *Vibrio* strains based on amplification of the toxin genes *tcpA* and *ctxA*. Grant, Hu, and Jinneman (2006) modified the real-time PCR technique to simultaneously detect heat-stable and labile toxin genes of enterotoxigenic *E. coli* with threshold cycles of 25.2–41.1.

A primary limitation of PCR has been that it could not distinguish between live and dead cells. However, newer PCR protocols have been designed that can target viable cells by detection of mRNA which is a marker of viability (Klein & Juneja, 1997; McIngvale, Elhanafi, & Drake, 2002; Morin, Gong, & Li, 2004).

Overall, PCR has tremendous power as a molecular profiling tool especially as a means to determine a pathogen's traits or to define its pathotype. It can also be useful for clustering gene traits and can provide significant information when bundled with analysis software to sort organisms into cluster groups or by trait possession (Rodriguez-Siek et al., 2005; Rodriguez-Siek, Giddings, Doetkott, Johnson, & Nolan, 2005).

Randomly Amplified Polymorphic DNA

RAPD is a PCR-based typing technique. Primers used in RAPD are relatively short in length (about 9–10 m). The sequences of the primers are random and capable of targeting nucleic acid dispersed throughout the genome for amplification to generate DNA fingerprints (Versalovic, Koeuth, & Lupski, 1991). Because of their small size, the primers are likely to bind to multiple sites on the genome DNA of interest. To facilitate primer annealing, the reaction temperatures are lower than those of normal PCRs (Williams, Kubelik, Livak, Rafalski, & Tingey, 1990). RAPD amplifies fragments of 200–2000 bp. Following amplification, products are separated by gel electrophoresis generating a series of banding patterns or "fingerprints". A drawback of RAPD is that it is difficult to guarantee reproducible results because of its random nature, and some researchers have found that lack of reproducibility limits its use on a broad scale. Williams et al. (1990) suggested that RAPD was useful for construction of genetic maps of inheritance. Lim, Lee, Hong, Bahk, and Choi (2005) used RAPD to differentiate *Salmonella* spp. using three different primers and compared these results to those obtained using other typing methods including enterobacterial repetitive intergenic consensus (ERIC) and single-strand conformation polymorphism (SSCP) methods. RAPD primers generated 42, 51, and 54 fingerprint patterns from 57 test strains, while ERIC produced 50 patterns, ribotyping PCR generated 4 patterns, and SSCP produced 11 patterns. The authors suggested that a combination of two methods (RAPD and ERIC) was necessary to ensure full differentiation of selected strains. Albufera, Bhugaloo-Vial, Issack, and Jaufeerally-Fakim (2009) reported that repetitive extragenic palindromic (REP) PCR gave better differentiation of closely related strains than RAPD and produced more complex banding patterns.

Repetitive Extragenic Palindromic PCR

REP PCR is based on the use of repetitive elements, such as repetitive extragenic palindromic (REP) elements, which are commonly found in bacterial genomes. This group consists of enterobacterial repetitive intergenic consensus (ERIC) sequences, *Salmonella* serotype enteriditis repeat elements (SERE), and

the BOX elements (Albufera et al., 2009; Hulton, Higgins, & Sharp, 1991; Rajashekara et al., 1998; Rasschaert et al., 2005; Versalovic et al., 1991). REP PCR primers are usually complementary to naturally occurring highly conserved DNA sequences (Rasschaert et al., 2005). REP and ERIC have been used in subtyping both Gram-negative and Gram-positive bacteria. PCR primers of REP and ERIC are designed to be homologous to specific REP and ERIC sequences. Amplification needs to be within the limit of the polymerase extension (about 5 kb). Test strains must have intergenic regions located on the chromosome which will result in patterns when the amplified product is run on a gel. A limitation of both REP and ERIC PCR is that they suffer from variation which makes it difficult to accurately reproduce the data from a reaction. The method has, however, been successfully used in discrimination of *Salmonella Enterica* at serotype level (Rasschaert et al., 2005). Here, the authors found poor banding patterns between different PCR runs but correlations were high within the same PCR runs. The authors also noted that one serotype did not correspond to one ERIC fingerprint; however, the method did allow subtyping of *Listeria* in bulk tank milk. Van Kessel, Karns, Gorsji, and Perdue (2005) suggested REP PCR is suitable for small tracking events (small niches). Other researchers have used REP PCR for poultry *E. coli* (Joerger & Ross, 2005) and differentiation of *Salmonella* Typhimurium from human and animal hosts (Woo & Lee, 2006). Goldberg, Gillespie, and Singer (2006) used one REP PCR primer and found high correspondence between REP and MLST profiles. In contrast, Foley et al. (2006) found no correlation among the results of REP PCR, pulsed field gel electrophoresis (PFGE), and MLST for isolates of *S. enterica* serovar Typhimurium recovered from humans and animals, while a similar study for *E. coli* O157:H7 (Foley et al., 2004) found that REP PCR generated a greater number of profiles for the pathogen from animals and humans compared to MLST (13 groupings versus 5 MLST types), but was limited compared to PFGE which generated 72 distinct profiles. Other researchers have found poor correlation between REP PCR and other molecular methods (Duirez & Topp, 2007; Mohapatra, Broersma, Nordin, & Mazumder, 2007; Ross, Merz, Farkosh, & Carroll, 2005; Sabat, Malachowa, Miedzobrodzki, & Hryniewwicz, 2006), while Albufera et al. (2009) found REP PCR showed greater discriminatory power in differentiating among closely related strains of *Salmonella,* as compared to RAPD, and produced more complex banding patterns.

Enterobacterial Repetitive Intergenic Consensus PCR

ERIC PCR works on a similar principle to REP PCR and is based on the amplification of enterobacterial repetitive intergenic consensus sequences (ERIC). Though both ERIC and REP PCR have been used successfully in characterization/differentiation of pathogens such as *E. coli*, *Listeria*, and *Campylobacter* (Albufera et al., 2009; Chou & Wang, 2006; Da Silveria et al., 2002; Hahm, Maldonado, Schreiber, Bhunia, & Nakatsu, 2003; Hiett, Seal, &

Siragusa, 2006; Jersek et al., 1999; Mohapatra, Broersma, & Mazumder, 2007), this method does appear to have similar drawbacks to REP PCR in that it suffers from considerable variation. In fact, variation has been observed between runs and between machines used to carry out the PCR, as well as day-to-day variation, making comparison of strains using ERIC PCR problematic.

Polymorphic Amplified Typing Sequences

PATS is a method developed for subtyping *E. coli* O157:H7 (Kudva et al., 2002). This technique is based on a similar principle to that underlying PFGE, where strains of O157 usually differ by a series of insertions or deletions, some of which are recognition sites of the restriction enzyme XbaI. Kudva et al. (2002) used these differences as a means to design a strain typing protocol that uses PATS. Primer sets were designed to amplify genomic DNA flanking the individual XbaI restriction sites located on reference genomes. This method is relatively similar to PFGE, but resolution is comparatively simple and less time consuming than PFGE. The method was able to identify polymorphic regions and identify isolates from the same outbreak as similar or identical. The PATS method was also capable of identifying three strains that were untypeable by PFGE. However, PATS was found to have lower sensitivity than PFGE when applied to discrimination between outbreaks, suggesting its most appropriate application may be in local epidemiology.

Variable Number Tandem Repeat—(VNTR) and Multi-locus Variable Number Tandem Repeat Analysis (MLVA)

Bacteria possess multiple regions or loci of repetitive DNA in their genomes. Within a bacterial genome, these areas of repeats vary in size and location (Lindstedt, 2005). The number of these repeat units per locus is a "strain-defining" parameter (Van Belkum, 2007). These regions are called variable number of tandem repeat regions (VNTRs). Differences in VNTRs can be exploited as these are specific in strains of a bacterial species (van Belkum, 2007). Assessment of the loci for variability is called multi-locus variable number of tandem repeat analysis (MLVA). MLVA can be used to trace isolates associated with outbreaks and is a versatile typing methodology.

Tandem finder software is used to design primers targeting these repeating elements. Primers amplify these loci by PCR, and the fragment size for each locus is determined using sequencing. Analysis of the fragment is assigned a variant score. MVLA types are assigned based on the compilation of all loci analyzed. Data from this analysis can be further analyzed using software to determine clusters, by generating dendrograms or spanning trees (Boxrud et al., 2007).

Sequence analysis also allows determination of mutations that are introduced into the region analyzed as well as assess the number of repeat units present.

VNTR has been used in analysis of a series of pathogens including *Bacillus anthracis*, *Yersinia pestis*, *Salmonella* spp., and *E. coli* O157 (Lindstedt, 2005).

Lindstedt, Heir, Gjernes, Vardund, and Kappered (2003) used MLVA to fingerprint *E. coli* O157 strains and found that the method was able to discriminate among strains. The method was considered relatively robust and fast. Among the 73 strains analyzed, 47 distinct profiles were found. When the method was compared with PFGE, high rates of co-clustering of MLVA and PFGE results occurred. In a recent review, Van Belkum (2007) discussed the use of MLVA for tracing isolates of a bacterial species, such as *S. aureus*, *Mycobacterium tuberculosis*, *B. anthracis*, *Y. pestis*, *E. coli*, *C. difficile*, and *S. enterica*, and they reported that the method could be used for assessing other systems such as viruses, fungi, and parasites. However, to the authors' knowledge, this method has not been tested for these types of organisms. MLVA has been used in subtyping a range of organisms including *S. aureus* and *M. tuberculosis*; bioterrorism agents; and enteric pathogens such as *E. coli* O157:H7 (Hoffmaster, Fitzgerald, Ribot, Mayer, & Popovic, 2002; Nøller, McEllistrem, Pacheco, Boxrud, & Harrison, 2003; Vogler et al., 2006), *C. difficile*, (Marsh et al., 2006; van den Berg, Schaap, Templeton, Klaassen, & Kuijper, 2007), *Salmonella* spp. (Boxrud et al., 2007; Torpdahl, Skov, Sandvang, & Baggesen, 2006), and *Shigella* (Lopez, Hilaire, Lisanti, Ramisse, & Vergnaud, 2008).

Restriction Endonuclease-Based Methods

Pulsed Field Gel Electrophoresis

PFGE has become a widely used method in molecular subtyping. It has been used extensively in the analysis of isolates associated with outbreaks such as *E. coli* O157 and *Salmonella*. PFGE was selected as the method of choice for PulseNet, the national system used for monitoring and tracking foodborne pathogens and outbreaks in the United States (Swaminathan, Barrett, Hunter, Tauxe, & The CDC PulseNet Task Force, 2001). This database is linked to the Centers for Disease Control. A range of PFGE protocols for pathogens such as *Salmonella*, *Listeria*, *Shigella*, *Campylobacter, Vibrio, Yersinia,* and *E. coli* O157:H7 are available through PulseNet (http://www.cdc.gov/pulsenet/protocols.htm).

PFGE involves the use of restriction enzymes to digest a pathogen's total DNA into fragments. Bacterial cells of interest are suspended in an agarose plug with an optimum number of cells (10^8) required to ensure quality fingerprints. Cells are lysed in the agarose plugs with proteinase K, sarcosine, and detergents, leaving the DNA intact. Digestion products, detergents, and other contaminants are removed from the plugs by a series of washing steps. DNA in plugs is then digested with restriction enzymes that are considered to be "rare cutting". That is, they will cut the DNA into fragments ranging from 10 to 800 kb in size. Typical enzymes used in PFGE include XbaI (Foley et al., 2004; Liebana, Garcia-Migura, Breslin, Davies, & Woodward, 2001), AscI, ApaI (Graves

et al., 2005), SalI, or SmaI (Suzuki, Ishihara, Saito, Ishikawa, & Yokochi, 1994; Wassenaar, Geilhausen, & Newell, 1998). There are a considerable number of restriction enzymes available for PFGE, and the reader is advised to choose enzymes based on the strain type and information desired. Restriction fragments are separated by gel electrophoresis in a specialized electrophoresis rig, as the fragments of DNA to be separated in this case are usually larger than those associated with other methods. PFGE depends on periodic inversion of the electric field in order that the strands of DNA trapped in the agarose reorient facilitating their movement through the gel. Most PFGE rigs use an array of electrodes arranged in a hexagonal formation, which generate electric fields at 120° to each other. Depending on size, DNA fragments move through the gel in a uniform manner, with the smaller fragments moving at a significantly faster rate than the larger ones resulting in a banding pattern (Carle, Frank, & Olson, 1986; Chu, Vollrath, & Davis, 1986).

PFGE is run in a specially designed rig with gel electrophoresis taking 18–24 h per run. Following electrophoresis, the DNA in the gel is stained with ethidium bromide to visualize the fingerprint. Images of the banding patterns are usually acquired electronically and imported into molecular analysis software such as BioNumerics® to generate cluster or dendrogram analyses useful in determining strain relatedness. Analysis software allows comparison of banding patterns and is useful in epidemiological investigations (see Fig. 18.2). One drawback of PFGE is that changes can occur in the banding patterns of strains during outbreaks as a

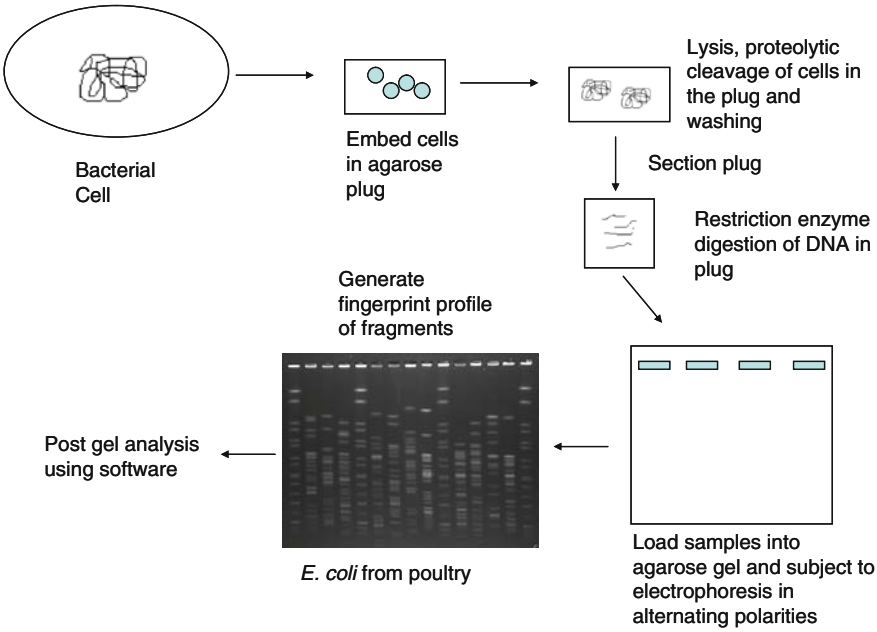


Fig. 18.2 Pulsed field gel electrophoresis

result of repeated subculturing or on passage of a pathogen through the intestine of the host (Hanninen, Hakkinen, & Rautelin, 1999; Iguchi et al., 2002; On, 1998; Steinbruckner, Ruberg, & Kist, 2001). Also, Kudva et al. (2002) found that PFGE patterns of *E. coli* O157 strains differed as a result of insertions and deletions in their O islands. PFGE has, however, proven valuable in outbreak investigations when used as a means to link pathogen to host, sources, and vehicles. However, PFGE may not be as powerful in some situations where the diversity of the strains is great. In such cases, there may be too many banding patterns or profiles to form conclusions. Li et al. (2007) used PFGE to subtype 138 *E. coli* recovered from processed bison carcasses and found 96 distinct banding patterns among the isolates tested. When further analysis investigated PFGE patterns in relation to the antimicrobial resistance of the strains, no correlation was found between resistance phenotype and genotype, with the 23 strains tested exhibiting 22 distinct patterns. PFGE has greater discriminatory index when used in application for distinct outbreaks or more specific pathogen types as opposed to generic *E. coli*. In contrast, similar application of PFGE for *Salmonella* isolates recovered from a poultry slaughter line (Nde, Sherwood, Doetkott, & Logue, 2006) was useful in demonstrating the movement of strains through the line and the effect of defeathering (Nde, McEvoy, Sherwood, & Logue, 2007) on carcass contamination. PFGE also has been reported to provide better discrimination in the molecular analysis of *S.* Typhimurium than MLST (Fakhr, Nolan, & Logue, 2005). Others have found that PFGE is useful for strain discrimination in outbreak situations or in surveillance associated with *Campylobacter* and *Salmonella* (Bender et al., 2001; Fitzgerald et al., 2001; Gilpin et al., 2006; Suzuki et al., 1994). However, Hedberg et al. (2001) considered PFGE unsuitable for routine typing of strains. Foley et al. (2004) compared PFGE with MLST and REP PCR for *E. coli* O157 analysis and found that PFGE provided the greatest discrimination. PFGE has been used in investigations of *Listeria* (Graves et al., 2005; Sanders et al., 2003) and *Campylobacter* (Wang & Taylor, 1990; Wassenaar et al., 1998). Ronner, Borch, and Kaijser (2005) used PFGE to trace/link human campylobacteriosis in Sweden with isolates from Thailand. Of interest was the finding of similar profiles in isolates from both countries demonstrating the global distribution of some *Campylobacter* strains. No relationship was found between PFGE profiles and antimicrobial sensitivities. Suzuki et al. (1994) investigated PFGE as a method to discriminate *C. jejuni* from sporadic infections with isolates from outbreaks. They found differences in cleavage patterns among isolates from sporadic outbreaks, which were more heterogeneous in nature than those from outbreaks which showed the same unique restriction patterns.

Restriction Fragment Length Polymorphism

RFLP is based on restriction analysis of individual genes that are amplified by PCR. The method can be used in the identification of a strain product or as a means to subtype variants of the same strain carrying the gene of interest.

Restriction enzymes are employed to cut the gene product of PCR into fragments of known size based on the DNA sequence of the gene. The fragments are run out on a gel using electrophoresis to generate restriction patterns or fingerprints. Pattern variation can arise if there are variants of the gene, or differences in intergenic regions, which will result in additions or removal of restriction sites (Pagotto et al., 2005). Pattern changes will therefore reflect differences in size and molecular weight of fragments (Pagotto et al., 2005).

RFLP has a variety of applications and offers the advantages of being faster and technically simpler to perform than PFGE. It has been used in ribotyping of the 16S or 21S rRNA spacer regions of pathogens (Kostman et al., 1995), and despite the observation that PCR ribotyping was less discriminatory than conventional ribotyping (Severino, Darini, & Magalaes, 1999), it has been used successfully for discriminating *Listeria monocytogenes* serotypes (Sontakke & Farber, 1995). It has also been used for the analysis of individual rRNA genes of *C. jejuni* (Iriarte & Owen, 1996) but may not be as valuable if multiple copies of the gene exist.

RFLP has been successfully used in subtyping *C. jejuni* using the flagellar genes, *flaA* and *flaB* (Clark et al., 2003; Nachamkin, Bohachick, & Patton, 1993; Mohran et al., 1996; Petersen & Newell, 2001; Peterson & On, 2000) as PCR targets and such enzymes as DedI, EcorI, HinfI, or PslI to digest the amplicons. However, results of flagellar RFLP have limited correlation with *Campylobacter* heat-labile serotyping, as some serotypes were represented by more than one *flaA* type (Nachamkin, Ung, & Patton, 1996). PCR RFLP has also been used to type shiga toxin-producing *E. coli* (STEC) using *stx1* and *stx2* genes (Beutin et al., 2007; Johansen, Wasteson, Granum, & Brynestad, 2001) and for subtyping shiga toxin genes (Ziebell, Read, Johnson, & Gyles, 2002). D'Auga, Zabreovskaia, and Grimont (1998) used RFLP for typing *Salmonella* flagella genes but found that the flagellin gene patterns did not correlate well with flagella serotype agglutination data. Foley, Zhao, and Walker (2007) noted that RFLP has limited success in determining the genetic diversity of *Salmonella* and is unable to distinguish between serovars of *Salmonella*, thus limiting its use in typing or source tracking.

Insertion Sequence RFLP

IS RFLP is a type of RFLP used in *Salmonella* investigations that targets *IS200* (Olsen, Skov, Angen, Threlfall, & Bisgaard, 1997). *IS200* is located randomly in the *Salmonella* genome. Studies using primers targeting *IS200* produced an amplicon of about 700 bp in size (Amavisit, Markahm, Lightfoot, Whithear, & Browning, 2001; Millemann, Gaubert, Remy, & Colmin, 2000; Olsen et al., 1997). Since *IS200* is not present in all *Salmonella* spp., it cannot be considered a dependable target for *Salmonella* analysis. Amavisit et al. (2001) noted that *IS200* profiles were indistinguishable among 28 *Salmonella* Heidelberg isolates from an equine veterinary hospital when compared to PFGE where the discriminatory power of PFGE was found to be significantly greater. In contrast,

Millemann et al. (2000) found that *IS200* PCR was relatively straightforward and provided good discrimination of *S*. Typhimurium isolates of bovine origin. Furthermore, the results of *IS200* PCR typing correlated well with other subtyping methods including RAPD PCR, ERIC PCR, and PCR ribotyping. Similarly, Olsen et al. (1997) noted that *IS200* PCR gave the best representation of overall similarity between *S*. Typhimurium isolates of human, pork, avian, and bovine origin. Regardless of its utility in certain situations, *IS200* PCR has been largely superseded by other molecular techniques such as REP PCR.

Ribotyping is an RFLP-based method that uses ribosomal RNA to subtype isolates of interest. In ribotyping, the DNA of the bacterial cell is digested with a frequent-cutting enzyme, and the fragments subjected to electrophoresis. Fragments in the gel are then transferred to a nylon membrane and hybridized with probes homologous to the conserved regions of the rRNA using Southern blotting (Chisholm, Crichton, Knight, & Old, 1999). Differences in the regions flanking the rRNA genes are reflected in variability in the size of fragments and in restriction patterns, which can be exploited to discriminate between strains. Ribotyping is a relatively reproducible method that can be automated (DeCesare, Bruce, Dambaugh, Guerzoni, & Wiedmann, 2001; Fontana, Stout, Bolstroff, & Timeeri, 2003; McCrea, Macklin, Norton, Hess, & Bilgili, 2006), and it generates few bands, making interpretation of results and strain differentiation relatively easy (Bailey et al., 2002). There are, however, limitations to the usefulness of ribotyping. In strains where the number of rRNA genes is low, ribotyping may be of little use (Foley et al., 2007). Also, for ribotyping to be useful in subtyping, mutations need to be in a location that influences the fragment size of the rRNA genes thus causing restriction pattern changes. Problems associated with methylation of nucleotides are also an issue in ribotyping and may result in poor restriction and affect banding patterns (Olive & Bean, 1999). Wassenaar and Newell (2000) also noted limited utility of ribotyping for *Campylobacter*. Although the method has a high level of typeability, most *Campylobacter* species contain three ribosomal gene copies, thus limiting the discriminatory power of the method and may also result in a limited ability to distinguish between strains of some *Campylobacter* spp. Kumao, Ba-Thein, and Hayashi (2002) suggested that ribotyping may not be useful in differentiating unrelated isolates within the same serotype. Ribotyping has, however, proven valuable in subtyping a range of foodborne pathogens including *Salmonella* (Fontana et al., 2003; Lim et al., 2005; Ling et al., 2000; Nayak et al., 2004; Tatavarthy et al., 2006), *Shigella* (Coimbria, Nicastro, Gimont, & Grimont, 2001; Lee et al., 2000; Liu et al., 1995; Rolland, Lambert-Zechovsky, & Denamur, 1998), *Campylobacter* (Fitzgerald, Qwen, & Stanley, 1996; Ge et al., 2006; Jackson, Fox, Waering, Hutchinson, & Jones, 1996; McCrea et al., 2006; Nielsen, Fusisng, Engberg, Nielsen, & Neimann, 2006), *E. coli* O157:H7 (Avery, Liebana, Reid, Woodward, & Buncic, 2002; Ito et al., 2003; Martin, Tyler, Khakhira, & Johnson, 1996; Richards et al., 2006), *Yersinia enterocolitica* (Blumberg, Kiehlbauch, & Wachsmuth, 1991; Iteman, Guiyoule, & Carniel, 1996; Mendoza, Alzugaray,

Landeras, & Gonzales-Hevia, 1996), and *L. monocytogenes* (DeCesare et al., 2001; DeCesare, Mioni, & Manfreda, 2007; Grif, Heller, Wagner, Dierich, & Wurzner, 2006; Nappi et al., 2005). Ribotyping has proven useful in outbreak investigations and in the evaluation of strains from human and non-human sources. The method works well in subtyping some strains or serovars but may not work well for all. The application of an automated version of the ribotyping technique is a valuable tool in situations where there is a need for high throughput of samples. The method is automated for lysis of cells, restriction of the DNA of interest, gel electrophoresis, and analysis against a known database. Automated ribotyping can handle about 32 samples in a working day (http://www.qualicon.com).

Amplified Fragment Length Polymorphism

AFLP is based on the amplification of restriction fragments of the genome of interest by PCR. In AFLP, the genome DNA of the strain of interest is digested with one or more enzymes, and the resulting fragments are ligated with known DNA fragments called linkers at the free DNA ends. The linkers serve as target DNA for the PCR primers to bind, thus allowing for selective amplification of specific fragments. The fragments are amplified by PCR, and the amplicons separated in a denaturing polyacrylamide gel or an agarose gel using electrophoresis (Savelkoul et al., 1999; Torpdahl et al., 2005; Vos et al., 1995). The fingerprints thus generated can be compared to other strains. The technique is relatively simple to perform, has high reproducibility, and is capable of differentiating between clones of strains.

As an alternative to gel electrophoresis, fluorescent-labeled primers can be used in place of standard primers, allowing detection of the fragments using an automated DNA sequencer (De Boer et al., 2000; Lindstedt, Heir, Vardund, & Kapperud, 2000; Tamada et al., 2001). Because a large number of restriction fragments are generated, PCR primers are designed to contain one to three additional nucleotides on the 3′ end extending into the unknown region of the DNA. Thus, efficiency of the primers becomes reduced by a factor of 4 for each additional nucleotide added to the primers which does not complement the sequence being amplified. This issue will result in less product being amplified and less bands generated. After amplification, the resulting products are separated on a DNA sequencing gel and an elution profile generated from fluorescent intensity associated with the labeled primers. AFLP in essence uses a selective amplification process to amplify specific fragments of the genome DNA of interest (Pagotto et al., 2005).

Although AFLP is a random technique, the randomness of amplification is reduced by running the PCR under stringent conditions, which results in only specific fragments being amplified. The primers are designed to match to complementary linker DNA with additional nucleotides added. AFLP is a relatively good quality typing tool and provides quality amplification because the primers are designed against the linker DNA which in turn selects specific

DNA fragments for amplification. Automation of the process using a DNA sequencer results in a relatively rapid typing technique. However, the method does suffer from variation if the process is carried out on different sequencing machines or platforms (Foley et al., 2007). An advantage of this technique is its ability to detect polymorphisms at the whole genome level, but a disadvantage of AFLP is the necessity for the use of a DNA sequencer to ensure the best differentiation and analysis, which is considerably more difficult to achieve using manual analysis.

Because of the high sensitivity of AFLP to minor genetic differences in strains, it is likely to remain a key molecular tool for use in strain typing. When AFLP is compared with techniques such as RAPD and RFLP, it displays similar if not better performance, in terms of reproducibility, resolution, and labor. AFLP is also a useful tool for studies into the ecology, evolution, epidemiology, and comparative genotypes of pathogens (Mueller & Wolfenbarger, 1999; Pagotto et al., 2005; Schouls et al., 2003; Siemer, Nielsen, & On, 2005).

Infrequent Restriction Site PCR

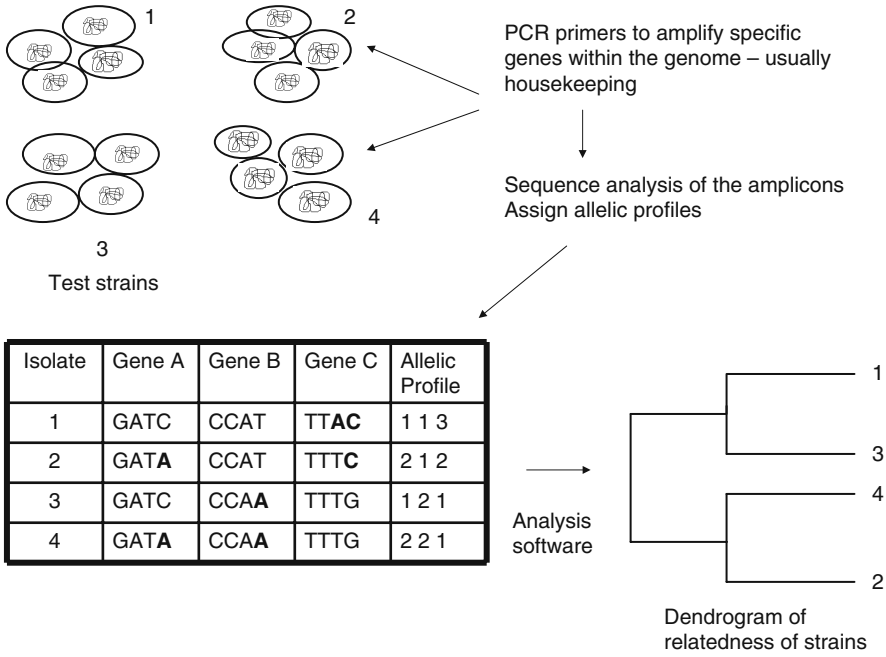
IR PCR was developed as a method to fingerprint isolates in epidemiological studies. The method is based on the amplification of DNA sequences flanking infrequent restriction sites followed by determining the electrophoretic patterns of the strains. In essence, the method is relatively similar to AFLP but differs in that it uses two enzymes to restrict the DNA of interest and two sets of linker DNA for each reaction. The restriction enzymes used include a frequent cutter and a less frequent cutter. This approach results in a large number of fragments. Fragments cut by both enzymes will be more important in IR PCR. Two rounds of PCR amplification follow cutting. In the first round, the linkers, attached at the target site of the infrequent cutter, are amplified, resulting in a strand that includes sequence complementary to the linker at the frequent cutter site. The second round of PCR amplifies linkers at the frequent cutter site, and amplification occurs from both sites. Therefore, amplification is relatively specific, depending on amplification from the infrequent site to result in further amplification (Mazurek, Reddy, Marstoin, Haas, & Crawford, 1996). This method has proven useful in epidemiological analysis, as it provides good levels of discrimination among strains (Mazurek et al., 1996). IR PCR has also been used in the investigation of *Salmonella* and *Listeria* spp. (Franciosa, Tartaro, Wedeli-Neergaard, & Aureli, 2001; Su et al., 2002). Garaizar et al. (2000) found that IR PCR had a high reproducibility rate but the discrimination index was low, the linkers in IR PCR help simplify the method and reduce the number of bands in a sample thus making analysis simpler. IR PCR was found to discriminate between serovars of *Salmonella* but was not as good at differentiating among strains of serovar Enteriditis. Su et al. (2002) found that IR PCR generated 7 profiles from 24 human *Salmonella* Enteriditis strains tested compared to 10 from PFGE. The authors commented that the IR PCR was a method of choice for large-scale epidemiological surveys due to its simplicity and its ability to differentiate among

clonally related strains. Franciosa et al. (2001) also noted the value of IR PCR for differentiation of *L. monocytogenes* associated with invasive and non-invasive disease with a diversity index of 0.919.

Sequence-Based Typing Methods

Multi-locus Sequence Typing

MLST is a nucleotide-based method for typing isolates. The technique is based on a similar principle to MLEE, where typing is based on the electrophoretic mobility of specific enzymes. MLST works on sequence analysis of specific housekeeping genes (Maiden et al., 1998; Turner & Feil, 2007). The method amplifies internal fragments of specific housekeeping genes, and then the amplicons are subjected to sequencing to determine the allelic profile of the isolate. Sequence differences are assigned an allele (see Fig. 18.3). Since MLST allows for variation in all possible genetic sequences, it is thought to be superior to PFGE in discriminatory ability (Enright & Spratt, 1999; Kotetishvili, Stine, Kreger, Morris, & Sulakvelidze, 2002). However, in some cases, the opposite appears to be true. For instance, in our lab, PFGE provided greater discrimination than MLST among *S.* Typhimurium from animals (Fakhr et al., 2005).



Isolate	Gene A	Gene B	Gene C	Allelic Profile
1	GATC	CCAT	TT**AC**	1 1 3
2	GAT**A**	CCAT	TTT**C**	2 1 2
3	GATC	CCA**A**	TTTG	1 2 1
4	GAT**A**	CCA**A**	TTTG	2 2 1

Fig. 18.3 Multi-locus sequence typing

It was suggested that the housekeeping genes sequenced were too conserved, with minimal variation observed. To enhance MLST's discriminatory value in this case, the authors proposed targeting genes in which greater variation could be expected, such as those associated with virulence. MLST has a distinct advantage over other molecular subtyping methods, as the data MLST generate, are easy to share for comparative purposes, and there is little interlaboratory or procedure variation that must be taken into account. A disadvantage of MLST is that housekeeping genes are relatively common and conserved and may be too conserved to provide good discrimination. Also, the value of this technique in an outbreak investigation may be questionable, as it requires sequence analysis, which would take time, thus delaying potential decisions.

MLST has been designed and used in a variety of pathogen investigations including *Salmonella* (Fakhr et al., 2005; Harbottle, White, McDermott, Walker, & Zhao, 2006; Hu, Lan, & Reeves, 2006; Kotetishvili et al., 2002; Torpdahl et al., 2005); *E. coli* and *E. coli* O157:H7 (Adiri, Gophna, & Ron, 2003; Nøller et al., 2003; Tarr et al., 2002); *Listeria* (Revazishvili et al., 2004; Salcedo, Arreaza, Alcala, de la Fuente, Vazquez, 2003; Zhang, Jayarao, & Knabel, 2004); and *Campylobacter* (Clark et al., 2005; Dingle et al., 2001; Litrup, Torpdahl, & Nielsen, 2007; Sails, Swaminathan, & Fields, 2003).

A variation on MLST which has been used to subtype pathogens is the application of MLST for virulence genes rather than housekeeping ones. This subtyping scheme is referred to as multi-virulence loci sequence typing (MVLST) and has proven useful in differentiating clones of *Listeria* associated with food outbreaks (Chen, Zhang, & Knabel, 2005, 2007; Zhang et al., 2004).

Single Nucleotide Polymorphism Analysis

SNP is a common method for analysis of eukaryotes. With the advent of genome sequencing of prokaryotes, SNP analysis has also been employed for the characterization and differentiation of bacterial strains. SNP uses nucleotide mutations in hypervariable loci as a means to differentiate strains. These variations may lead to changes in amino acid profiles (Foley et al., 2007). Mutations that are present in a genome can be used to provide evolutionary origin information and to distinguish among closely related strains. Cebula, Jackson, Brown, Goswami, & LeClerc (2005) suggested that multiple SNPs could be used to determine the relatedness of strains. This can be achieved by selecting genes with known high polymorphism rates or by mining genome data to generate microarray probes corresponding to each of the nucleotides of the potential gene SNP fragments. DNA from multiple bacterial species is incubated with the array and hybridization is used to detect loci that are polymorphic. Identified loci for SNPs are sequenced to find the nucleotide position that is polymorphic. Multiple methods can be used to detect polymorphisms at SNP locations, including DNA sequence of the region. Other methods to detect SNPs include RFLP, mass spectrometry, real-time PCR, microarray analysis,

and flow cytometry (Foley et al., 2007). SNP analysis has been used in studies of quinolone resistance and flagellar antigens of *Salmonella,* as a means for phylotyping isolates of *E. coli*, and in the analysis of *S. aureus* and *Neisseria meningitidis* (Esaki et al., 2004; Hommais, Pereira, Acquaviva, Escobar-Paramo, & Denamur, 2005; Levy, Sharma, & Cebula, 2004; Mortimer, Peters, Gharbia, Logan, & Arnold, 2004; Robertson et al., 2004).

Genome Sequencing and Comparative Genomics

Whole genome studies of foodborne pathogens are becoming significantly more accessible, as rapid advances in whole genome sequencing technology have been accompanied by substantially reduced sequencing costs. Consequently, the numbers of completed genomes and those undergoing sequencing are increasing rapidly. Currently, the Genomes Online Database (GOLD) (http://www.genomesonline.org/), which monitors genomes being sequenced worldwide, lists 762 completed genomes (of these 627 are bacterial genomes) and 1749 bacterial genomes as currently undergoing sequencing (as of April 14, 2008). Availability of these genomes through online sites and publications greatly facilitates high throughput analysis of organisms of interest, allowing significant insights into all aspects of pathogenesis, virulence, gene regulation, antimicrobial resistance, gene transfer, microbial metabolism, and so on. Comparative genomics allows researchers to compare genomes of similar or even nonsimilar strains in order to understand their relationship on a fundamental level. Johnson, Kariyawasam, et al. (2007) used comparative genomics to study the purported link between avian pathogenic *E. coli* and human extraintestinal pathogenic *E. coli*, which cause human urinary tract infections, sepsis, and neonatal bacterial meningitis. Similar comparisons are common through online databases designed for whole or partial genome analysis. Chaudhuri, Khan, and Pallen (2004) described the development and use of an online database for online comparison of *E. coli*, *Shigella*, and *Salmonella* (http://colibase.bham.ac.uk). Similar databases have also been developed for *Clostridium* (http://clostri.bham.ac.uk) and *Campylobacter* (http://xbase.bham.ac.uk/campydb/). Of particular interest in the development of such databases is the ability to compare genomes and determine protein coding without functionality assays (Pagotto et al., 2005).

Genome-wide analysis techniques generate massive amounts of data that can be compared to reference genomes using alignment software. Such software tools include those developed on specific sites such as *coli*BASE or through public search tools such as Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI). When bioinformatics is applied further it can be expanded and used in studying the functional genomics of the strain and has application in gene expression analysis using technology such as microarrays.

Hybridization Techniques

DNA Microarrays

DNA microarrays consist of gene probes arrayed on a substrate. Such arrays may be limited to select genes of interest or be multi-genome-wide in scope. Among other things, they can be used for comparative genomic hybridization (CGH) (also called genomotyping) and to study genome-wide gene expression. They have quickly become a powerful tool in genomic analysis of pathogens. The microarray itself (often called a gene chip or biochip) is a series of DNA molecules of known sequence called probes that are fixed to a substrate (usually a special type slide). These probes consist of partial gene sequences, generated from PCR, full-length cDNA, or oligonucleotides (Pagotto et al., 2005). Such microarrays can be used to compare strains at a genome-wide level (CGH). Alternatively, they can be used to perform expression studies of the whole genome or select genes. A recent review by Ojha and Kostrzynska (2008) highlights the application of microarray technology in the field of veterinary research for pathogen infection investigations, diagnostics, and studies of host pathogen interactions. Similar research by Jin, Tao, Li, Li, and Li (2005) used microarray technology to investigate *E. coli* O157:H7.

Often, microarray analysis is used to determine gene expression under differential conditions. Such techniques are fundamental to identification of bacterial virulence mechanisms, as virulence genes are likely to be expressed during infection or under host conditions (White-Ziegler, Malhowski, & Young, 2007). In such cases, one sample of the bacterium of interest is exposed to standard conditions, while another sample is grown in the host or under conditions mimicking those of the host. Free nucleic acid is prepared from mRNA of strains in the control and treatment groups and is differentially labeled with fluorescent dyes (Cy3 and Cy5 are the most common). Then the labeled nucleic acid is added to the array and allowed to hybridize. Automated versions of hybridization use a cassette to cover the whole chip and employ automatic washing stages to rinse the slides with the samples of interest. Such automation reduces human error and allows the conditions of the hybridization to be strictly controlled for temperature during rinsing stages. Hybridization results are then read using a two-color confocal scanner to detect fluorescence at the wavelengths of the two fluorescent dyes (550 nm for Cy3 and 650 nm for Cy5). The resulting fluorescence is measured and quantified for analysis. Depending on the colors and intensity of each spot it is possible to determine the expression level of the genes associated with the spot and consequently which genes that are up- or downregulated in response to the "treatment" (see Fig. 18.4).

Microarrays can also be used to assess similarities between strains, characterize strains or subtype strains. Boyd, Blackmer, and McCelland (2003), Gaynor et al. (2004), Hain, Steinweg, and Chakraborty (2006), Malik-Kale et al. (2007), Parker Miller, Horn, and Lastovica (2007), and Raengpradub,

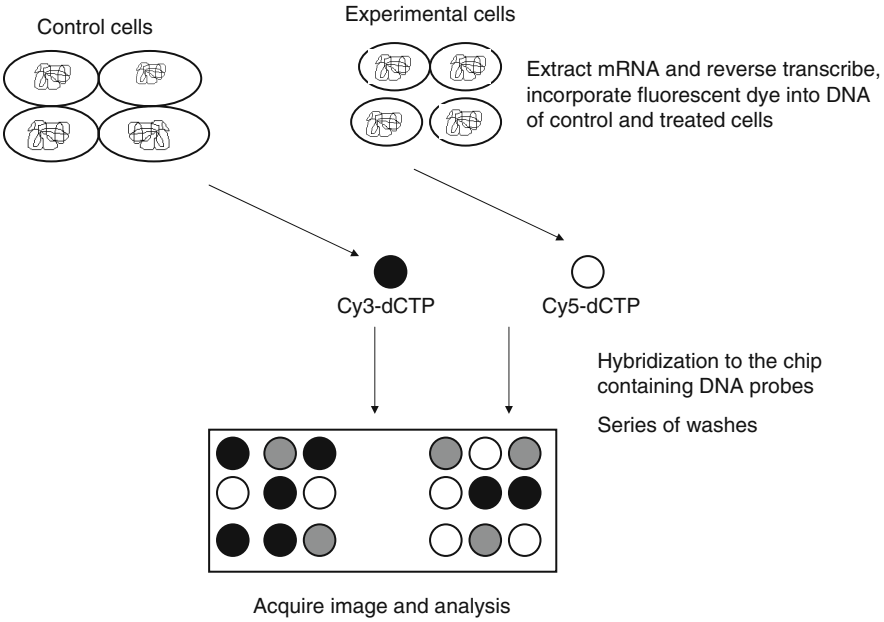


Fig. 18.4 Microarray analysis of gene expression

Wiedemann, and Boor (2008) have described the use of microarray technology to analyze strains at the genetic level for comparative purposes. Anjum et al. (2007), Batchelor et al. (2008), Chen et al. (2005), Garaizar, Rementeria, and Porwollik (2006), Reen et al. (2005), Volokhov, Rasooly, Chumakov, and Chizhikov (2002), Volokhov, Chizhikov, Chumakov, and Rasooly (2003), Yoshida et al. (2007), and Zhang et al. (2007) have used microarrays to identify various bacteria to species or subspecies level, detection of virulence or antimicrobial resistance genes and Call, Brockman, and Chandler (2001), Chandler et al. (2001), Keramas et al. (2004), Kostic et al. (2007), Kostrzynska and Bachand (2006), Quinones, Parker, Janda, Miller, and Mandrell (2007) have used them for detection, identification, and characterization of pathogens in a range of samples. Given the unlimited amount of information available, microarrays can be designed and built for a range of purposes such as determining expression of specific virulence or antimicrobial resistance genes or for more specific processes, such as study of invasion, flagellar production, growth processes, or biofilm production. DNA microarrays offer much promise for future studies in understanding pathogens, hosts, and production systems—they can be used to model host–pathogen interactions and the effect of various drugs or vaccines on a host or pathogen. Therefore, it is likely that microarray technology will provide needed insight into the mechanisms of pathogenesis used by foodborne pathogens that can be exploited to make food safer.

Conclusions

Molecular methods offer scientists the opportunity to explore the food safety continuum as never before. Yet these methods have different strengths and drawbacks, requiring careful choice of technique in order to ensure the value of the results generated. Techniques such as PCR and real-time PCR may be very valuable in rapid screening for particular genes but a technique such as PFGE may provide greater information about strain diversity. One cannot overlook the fact that in some instances a single molecular method may not be sufficient for useful strain differentiation. Costs of implementing molecular techniques may also be inhibitory. Certainly, sequencing and microarray technology, which have high initial capital investment and high running costs including those associated with labor, training, and upkeep, may not be suitable for routine situations. Alternatively, time may be of the essence in some cases, necessitating consideration of certain rapid molecular methods. Thus, a myriad of concerns must be taken into account when making a decision about a technique to be used.

Molecular methods have become a significant part of the food industry arena. They provide tools to allow better discrimination of pathogens and consequently are useful in epidemiological studies for the identification of foods, hosts, or environmental sources involved. Of concern in molecular methods is the true lack of a gold standard method that has broad application for all pathogens and that could be easily standardized among labs worldwide. As we look to the future, genomics-based methods show great potential for filling the niche in standardizing methods, and it will be interesting to see where the future of these types of applications will go. Regardless, we anticipate that for a molecular method to attain widespread use for food safety application it will need to be easy to perform, rapid, cost-effective, and have broad application for a range of pathogens, providing good discriminatory power and applicability.

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Chapter 19
Methodologies for the Detection of BSE Risk Material in Meat and Meat Products

Ernst Lücker

Introduction

Soon after the emergence of bovine spongiform encephalopathy (BSE), a fatal disease of the central nervous system (CNS) in cattle, so-called specified bovine offal were legally defined and banned (SBO-ban) in order to reduce the presumed potential BSE exposition risk for British consumers (UK, 1989). Later on the legal definition of risk material was frequently modified according to new scientific results on BSE tissue infectivity (Table 19.1). A European-wide ban on specified risk materials (SRM) was established in 2001 (EC, 2001). In effect, the SRM-ban is still the most important direct measure in reducing potential human BSE exposure risk (EC, 2005). Taking into account the overall and constant reduction of the frequency of BSE cases as well as the very high costs of preventive measures, the European Commission has envisioned a future lifting of the SRM-ban (EC, 2005). Scientific uncertainties and new insights into atypical BSE, however, do not argue in favour of a total lift of the ban (Kong et al., 2008), in particular without establishing alternative preventive measures. As such, suitable analytical methods for the detection of SRM will be needed in future. SRM are currently defined as listed in Table 19.2 according to EU-legislation (EC, 2001). From an analytical point of view this definition is extremely complex in encompassing not only a variety of different materials (such as skull, brain, intestine, tonsil) but also (i) the species, (ii) the age and (iii) the provenance of the animal from which the respective material was derived. To facilitate analytical detection, we can focus on tissues of the central nervous system (CNS) including peripheral nerve tissues closely located to the CNS such as dorsal root ganglia (Fries et al., 2003; Piske et al., 2007). Histopathological lesions are restricted to the CNS where massive PrP^{Sc} replication is correlated to increasing infectivity. From data of the Scientific Steering Committee of the

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Table 19.1 Total BSE infectivity in tissues of a clinically BSE affected bovine as estimated by the Scientific Steering Committee of the European Commission (SSC, 1999)

Tissue	Infectivity density ($CoID_{50}/g$[a])	Weight (kg) per 537 kg animal	ID_{50} per BSE case	% of total infective load per animal	Cumulative load
Brain	10	0.5	5000	64.1	64.1
Spinal cord	10	0.2	2000	25.6	89.7
Trigeminal ganglia	10	0.02	200	2.6	92.3
Dorsal root ganglia	10	0.03	300	3.8	96.1
Eyes	3.20E-02	0.1	3	0.04	99.1
Ileum	3.20E-01	0.8	260	3.3	99.4
Spleen	3.20E-02	0.8	26	0.3	99.7

[a] $CoID_{50}$: cattle orale infectious dose (50%).

Table 19.2 Specified risk material (SRM) according to Article 6 and Annex V of Directive (EC) 999/2001 (EC, 2001)

Species	Material	Age limit
Bovine animals	Skull (excluding mandible), brain, eyes, spinal cord, head meat[a]	> 12 months
	Vertebral column (excluding vertebrae of the tail, spinous, transverse processes of the cervical, thoracic and lumbar vertebrae, median sacral crest and wings of the sacrum), dorsal root ganglia	> 24 months
	Tonsils, intestines (from the duodenum to the rectum), mesentery, basis of tongue[b]	None (all ages)
Ovine and caprine animals	Skull, brain, eyes, tonsils, spinal cord	> 12 months
	Spleen, ileum	None (all ages)

[a] If not harvested in accordance with a control system, recognized by the competent authority, to ensure the prevention of possible contamination of head meat with central nervous system tissue.
[b] Tongues of bovine animals of all ages intended for human or animal consumption shall be harvested at the slaughterhouse by a transverse cut rostral to the lingual process of the basihyoid bone.

European Commission (SSC, 1999) it could be estimated that about 95% of total BSE infectivity is concentrated in tissues of the CNS (Table 19.1). More recently, the reduction of BSE infectivity by removal of CNS-based SRM was estimated to be in the range of up to three orders of magnitude (Lücker, 2006). In addition to the food safety aspect, methods for the detection of CNS contribute to the authorities' ability to detect and deter deviations from food labelling regulations (EC, 2000; Agazzi, Barrero-Moreno, Lücker, v. Holst, & Anklam, 2002). Since 1997, a wide community of scientists have contributed to the development of a panel of methods for the detection of CNS on meat and in meat products. The

current state is presented in the following as ordered by the nature of the CNS marker (lipids, proteins, nucleic acids) and, secondly, by methodological principal.

Markers from the Lipid Fraction

Certain fatty acids of the CNS promise to become important CNS markers showing a high potential for further analytical discrimination according to the complex legal definition of specified risk material. Although of minor interest in current method development, cholesterol will be included in the discussion as a very robust and easy-to-detect marker for CNS in meat products.

Cholesterol

Historically, cholesterol was the fist marker used to detect CNS in heat-treated meat products (Lücker & Bülte, 1997). The cholesterol content of the CNS exceeds that of the major meat technological relevant tissues, such as muscle or adipose tissues, by several orders of magnitude. For example, the cholesterol content in brain and muscle tissue is given as about 50 and 2000 mg/100 g (wet weight), respectively. Quantification can be easily achieved by use of an enzymatic photometric method. The test principle is based on the quantitative oxidation of the 3-ß hydroxy group of cholesterol to delta4-cholesten-3-on using the cholesterol oxidase which is followed by the photometric detection and quantification of a lutidine colour reaction at 405 nm (Lücker et al., 1999). At the time of the emergence of a new variant of CJD which confirmed the concerns about transmissibility of BSE to humans, the ready availability of CNS detection in meat products for official food control played a prominent role in method development. In fact, the principal method had already been validated for cholesterol quantification in eggs and egg-derived products, e.g. by the federal German health authority (BVL, 1992). Thus, only slight modifications of the method were needed. In order to account for the presence of cholesterol in non-CNS tissues, however, cut-off values for normal cholesterol contents in meat products (without addition of CNS) had to be established. In emulsion-type sausages it was possible to detect CNS at levels of about 1% with a 99.9% statistical security (Lücker et al., 1999), while cooked sausages, due to the addition of high amounts of cholesterol-containing liver, caused some problems (Lücker, Horlacher, & Eigenbrodt, 2001). When analysing sausages with addition of tissues with relevant cholesterol contents, such as liver, kidney or egg products, we have to expect either a higher count of false-positive samples or we apply a higher cut-off value which would then lead to an increased number of false-negative results. Overall, the cholesterol quantification as a method for the detection of CNS in meat products is of some interest,

not only for laboratories with basic instrumentation and low budgets but also as a secondary method supplementing fast immunological screening methods (see below) as pertaining to heat-treated samples with low but conspicuous results (Lücker et al., 2001). Moreover, cholesterol offers high potentials to be applied as CNS marker in a low-cost miniaturized rapid test system. This is particularly interesting for monitoring CNS contamination of raw meat in the slaughtering process where other matrices with high cholesterol content do not interfere.

Fatty Acids (FAs, FAMEs)

Sphingolipids, such as galactocerbrosides, gangliosides and sphingomyelins, constitute highly interesting markers for CNS in meat products, particularly with regard to their specificity for and high content in myelinized nervous tissue. In modern food control laboratories, methods for the detection of such compounds – such as gas or liquid chromatography and mass spectrometry – are readily available. A further advantage can be seen in the potential stability of most of the compounds from the lipid fraction as they are presumed to be much more stable than those of the protein or nucleic acid fraction of the CNS used as markers in other phenotypic or in genotypic methods. Niederer and Bollhalder (2001) were the first to make use of this novel analytical approach in CNS detection. They identified certain fatty acids within the complex lipid fraction, in particular nervonic acid, to be absolutely specific for CNS. Following solid-phase extraction and acid derivatization to the respective methyl esters, the FAME were separated and quantified by means of GC/MS. Detection limits were reported to be as low as 0.01% CNS in standards of meat products. They demonstrated that the relation between the *cis*- and *trans*-isomers of nervonic acid offered the possibility to categorize the detected CNS as pertaining to the species and age of the animal from which the detected CNS originated. Although first reproduction of the proposed method indicated that the general potential of this analytical approach was valid, certain drawbacks became apparent (Agazzi et al., 2003; Biedermann, Lücker, & Hensel, 2002; Lücker, Biedermann, Lachhab, Truyen, & Hensel, 2004; Noti, Biedermann-Brehm, Biedermann, & Grob, 2002). Mainly, most non-CNS tissues as used in meat technology showed base-line contents of the presumed CNS-specific FAs, including nervonic and cerebronic acid. Thus, it was necessary to establish cut-off values for the CNS-FAs as was the case with cholesterol. Fortunately, the specificity of CNS-FAs is still distinctly higher than that of cholesterol to the effect that detection limits can be achieved which are one to two orders of magnitude lower. Moreover, structural characterization studies by retention time and dimethyl disulfide adduct profiling proved the previously designated "trans-nervonic acid" (*trans*-15-tetracosenic acid) to be *cis*-17-tetracosenic acid (Biedermann et al., 2004). Nevertheless, small amounts of *trans* isomers of some long-chain FAs were detected which might improve efficiency of the

differentiation between non-SRM and SRM according to the legal definition. In a further study the ratio of ω 7-/ω 9-isomeres of tetracosenic acid ([15c-C24:1]/[17c-C24:1]) was shown to be well suited to differentiate between the non-SRM and SRM status of CNS traces in meat products (Lücker et al., 2004). Besides some methodological optimizations which resulted in the presentation of a standard operating procedure (SOP), the authors proposed an analytical strategy essential to the quantification of the CNS. It became apparent that the content of all relevant FAs (C22:6, C24:1 ω 9, C24:1 ω 7, C24:0, C24-OH) in the respective CNS varies as a function of species and age. This necessitates an analytical strategy which has to start with the non-specific identification of the presence of CNS in the sample (step 1). In case of CNS-positive samples, the second step involves the analytical identification of species and age of the CNS (step 2); only then does it become possible to quantify the CNS, as a species- and age-specific calibration has to be applied (step 3). Cerebronic acid turned out to be the most suitable FA for both CNS identification (step 1) and CNS quantification (step 3). Following sylilation, cerebronic acid showed maximum absolute differences as well as the best ratio between contents of CNS-free samples and CNS.

In spite of its analytical complexity it was demonstrated – within an externally controlled blind test – that the present approach using FAs as markers and GC/MS is a highly sensitive and robust method which facilitates the identification of the SRM status of CNS traces in meat products (Grießbach et al., 2007). Within the observed range of 0.5–3% CNS addition, neither the animal species of the main component (muscle tissue) nor severe heat treatment (up to 133°C, 3 bar, 40 min) had any influence on the capability to identify the SRM status of the detected CNS traces. Further research activities deal with optimizing the analytical procedure, in particular the sample extraction (Pörschmann, Trommler, Biedermann, Truyen, & Lücker, 2006), as well as the statistical data evaluation as pertaining to CNS-species and CNS-age differentiation, which depends on a comprehensive database of CNS-FAs and multifactorial data analyses of FA patterns (Grießbach et al., 2008). Pörschmann and co-workers (2006) demonstrated that sequential pressurized liquid extraction (PLE) can prove superior to the originally used exhaustive lipid extraction followed by solid-phase extraction (SPE) regarding lipid recoveries and clear-cut boundaries between lipid classes. Alcoholysis using trimethylchlorosilane/methanol facilitated complete transesterification of lipids and quantitative formation of methyl esters. Grießbach and co-workers (2008) showed that differentiation of CNS-species and CNS age must be founded on a comprehensive database of CNS-FAs and can be significantly enhanced by multifactorial data analyses of FA patterns.

For the time being, the analytical FA-GC/MS approach for the detection of CNS in meat products is the only method which enables us to differentiate between non-SRM and SRM status of the detected CNS insofar as animal species and age of the detected CNS can be discriminated. While, on the other hand, the analytical strategy and procedure is very complex, time and cost

intensive, it could be best put to use as future reference method, in particular to validate and more closely characterize positive results of immunochemical screening methods or within the scope of method development. Furthermore, the extraordinary heat resistance of these FA-CNS markers could constitute a basis for further research in characterizing CNS contamination not only in meat products but also in the meat and bone meals.

Markers from the Protein Fraction

Immunological detection of proteins as specific, albeit highly conserved, markers for CNS in meat products made possible the first detection of illegal use of brain in meat products.

Of special interest is the neuron-specific enolase, particularly from the historical point of view. This marker is thus mentioned prior to glial fibrillary protein, which currently can bee seen as the most important immunological CNS marker. Other proteins used in immunological test systems for the detection of CNS – but not exclusively in meat products – are mentioned separately. In addition to the purely immunological approach, a short review of immunohistological studies is given which importantly combines morphological and immunological information. Finally, the prion protein, not in its normal (cellular) structure – which is unspecific and thus unsuitable for our purposes – but in its abnormal isoform will be discussed. Although the method principle is comparable, the interpretation of the analytical result is totally different and presents an alternative approach to the question of how to detect BSE risk in meat products.

Neuron-Specific Enolase (NSE)

In the year 1998 the first immunological method for the detection of CNS in meat products was reported in literature (Lücker, Eigenbrodt, & Bülte, 1998). As main CNS marker the neuron-specific enolase (NSE, γ-enolase) was used. The method applied poly- later on monoclonal anti-NSE antibodies in a Western blot after protein extraction and SDS-PAGE separation (Lücker et al., 2001; Lücker, Eigenbrodt, Wenisch, Leiser, & Bülte, 2000). Detection limits – as demonstrated by blind testing using standard emulsion-type sausages with varying brain contents – were estimated to be as low as 0.01% CNS (Lücker et al, 2000). In combination with cholesterol quantification the NSE-Western blot was applied to a university-based low-scale screening of retail meat products from German food outlets. Already in May 1998 the first CNS-positive sample was detected. A high-quality tinned liver sausage product showed strong immunoreactions in the NSE-Western blot and also significantly increased cholesterol contents. The use of brain was not labelled. Repetition of analysis

in samples from different production batches was also positive. Official follow-up inquiries yielded the information from the manufacturer that indeed brain had been used, however, only porcine brain and in a quantity of about 4%. Further application of NSE-Western blot and cholesterol quantification to retail meat products demonstrated a much more frequent and widespread use of CNS in meat technology than was suspected at this time. Early on the NSE-Western blot was officially introduced in Swiss import control (Bissig-Choissat, Kuhn, Schlosser, & Jemmi, 2002) and later it was established in the United States (Salman, Jemmi, Triantis, & Dewell, 2005). In Germany, a commercial NSE-Western blot became available in 2001 (ScheBo-BioTec, Gießen, Germany). Several lab-internal (e.g. Lücker et al., 2001) and inter-laboratory validation studies were conducted using the NSE-Western blot, some of them in comparison with other CNS-detection methods, which became available in the meantime (Agazzi et al., 2002; Hajmeer, Cliver, & Provost, 2003; Hughson, Reece, Dennis, & Oehlschlaeger, 2003). Although all of these studies yielded good analytical results, the NSE-Western blot did not become very widely used. This can be primarily attributed to the fact that the Western blotting technique is not widely established in the laboratories of official food control and, thus, problems in basic applicability occurred. In particular the standardization of the analytical procedure and the interpretation of the immunoblots appeared not to be error proof. In addition, presumably false-negative results were reported for products containing avian matrices (Bissig-Choissat et al., 2002; Salman et al., 2005). Indeed, avian tissues can cause non-specific immunoreactions in close vicinity of the NSE-band (Schlottermüller & Lücker, 2002). In view of the superior practicability of the ELISA technique a survey of available and newly produced monoclonal anti-NSE-antibodies was conducted. This study, however, failed to identify suitable antibodies for the ELISA technique with respect to the detection of CNS from heat-treated matrices (unpublished data).

Glial Fibrillary Acidic Protein (GFAP)

The astrocytic glial fibrillary acidic protein (GFAP), a major protein constituent of glial filaments in differentiated astrocytes, was first introduced as marker of CNS for food control purposes in 1999 (Schmidt, Hossner, Yemm, Gould, & O'Callaghan, 1999). The study used a modification of a colorimetric enzyme immunoassay originally designed for clinical purposes (GFAP-ELISA) including a sandwich of polyclonal and monoclonal anti-GFAP antibodies for the detection of CNS in mixtures with fresh muscle tissue and blood. The limit for detection of GFAP was approximately 1.0 ng. Sensitivity was improved to 0.2 ng GFAP by introducing a fluorescent enzyme-linked detection system GFAP F-ELISA (Schmidt, Yemm, Childs, O'Callaghan, & Hossner, 2001). GFAP was not detected in skeletal muscle and blood clots and only in traces in

the sciatic nerve (ng/mg range) while CNS showed contents in the μg/g range (Schmidt et al., 1999). A commercial version of the GFAP assay became available in 2001 (R-Biopharm, Darmstadt, Germany). Applicability of the GFAP-ELISA was demonstrated also for heat-treated meat products, and validation studies including comparisons with other CNS markers and detection systems showed altogether good to excellent results (Agazzi et al., 2002; Agazzi, Barrero-Moreno, Lücker, v. Holst, & Anklam, 2004; Hajmeer et al, 2003; Hughson et al., 2003; Schmidt, Yemm, Childs, O`Callaghan, & Hossner, 2002; Rencova, 2005; Kale, Kursun, & Pehlivanoglu, 2007). The principle of CNS detection by means of GFAP-ELISA was validated and introduced as official method for food control in Germany in 2004 (BVL, 2004). Recent studies focused on the detection of non-heat-treated CNS such as contamination of fresh meat or of carcass-splitting band saw blade surfaces and ground meat or advanced meat recovery samples (Reddy et al., 2006; Hossner et al., 2006). In comparison the GFAP F-ELISA proved to be superior to the commercial GFAP-ELISA version and the immunohistochemical GFAP detection in terms of sensitivity and repeatability (Hossner et al., 2006). According to the authors all three methods are widely used in the United States. In contrast to these studies which demonstrated CNS specificity of GFAP, false-positive results were obtained in two cases (Schurr, Lücker, & Troeger, 2004; Kunath, Lücker, Troeger, & Grundmann, 2004). Blood samples taken *intra vitam* from clinically healthy cattle frequently showed positive immunoreactions in the range of up to 0.2% CNS using the commercial GFAP-ELISA. In contrast, these reactions were not detected when using other CNS-detection methods, in particular the laboratories own newly developed GFAP-ELISA (Kunath et al., 2004) which became commercially available in 2004 (ScheBo-BioTec, Gießen, Germany). Currently, the GFAP-ELISA can be seen as the standard procedure for CNS detection for screening of fresh and heated meat products and for the control of CNS contamination of surfaces of meat or equipment.

Other Proteins

While a large panel of antigens were immunologically tested for their suitability to detect CNS in fresh and heat-treated meat products (e.g. Overhoff & Lücker, 2003) only a few gave promising results. Myelin basic protein (MBP) was shown to be a suitable CNS marker involving immunochemical detection by an indirect ELISA technique (Holtbecker & Stolle, 2005) and by Western blot (Overhoff & Lücker, 2003; Herde, Bergmann, Lang, Leiser, & Wenisch, 2005). Moreover, these studies indicated MBP to be potentially species specific and thus enabling us to differentiate between bovine and porcine or avian CNS.

Myelin proteolipid protein (PLP) was identified as a further suitable CNS marker in meat products or on meat (PLP). Western blot analysis of PLP detected CNS contamination selectively and sensitively (Villmann, Sandmeier,

Seeber, Hannappel, Pischetsrieder, & Becker, 2007). Bäuerlein et al. (2008) developed a rapid dot blot assay using an anti-PLP antibody. The detection limit was reported as 0.01% fresh bovine brain in minced bovine muscle. The assay can be applied in a swab test allowing a detection of down to 0.5 mg CNS on meat or other surfaces. Protein gene product 9.5 (PGP 9.5) might become another suitable antigen for immunological CNS detection in heat-treated meat products as recent results on the development of a sandwich ELISA indicate (Gaunitz, Gabert, Lücker, Seeger, & Stahl, 2008).

Immunohistology

Immunohistological methods include micro-morphological criteria in addition to specific antigen detection. For instance, morphological criteria might facilitate the differentiation between central and peripheral nervous system (PNS) tissues when using antibodies of respective low specificity. Moreover, non-CNS risk materials, such as lymphatic tissues or intestines, can be detected by micro-morphological analysis (Koolmees, Tersteeg, Keizer, van den, & Bradley, 2004). The first immunohistologic approach for the detection for CNS demonstrated the suitability of NSE – in contrast to GFAP – in heat-treated meat products (Wenisch, Lücker, Eigenbrodt, Leiser, & Bülte, 1999). Another study which tested GFAP, NF, NSE and MBP (Tersteeg, Koolmees, & van Knapen., 2002) showed the interdependence of heat treatment and type of antibody/epitope used for the detection. Here, the selected anti-MBP antibody turned out to be of superior applicability. A further study showed an anti-NF antibody to be more suitable than antibodies against NSE, GFAP, MBP or peripherin (Aupperle, Lücker, Overhoff, & Schoon, 2002). NF facilitated the detection of 1% CNS addition in heat-treated meat products and differentiation between nerve fibres and CNS became possible by using morphological criteria. Currently the U.S. Department of Agriculture, Food Safety Inspection Service uses an immunohistological procedure for GFAP (USDA, 2003) which, however, was shown to produce inconsistent results (Hossner et al., 2006). Herde and co-workers (2005) report the suitability of certain anti-GFAP and anti-MBP antibodies for the detection of bovine brain (8%) in heat-treated meat products. Anti-synaptophysin antibodies were used for CNS detection in immunohistological studies with non-heated matrices (e.g. Collins Kelley, Hafner, McCaskey, Sutton, & Langheinrich, 2000) and anti-S100ß-protein immunostaining gave first proof for micro-embolization of brain tissues due to captive bolt stunning (Anil et al., 2001).

Prion Protein (PrP^C, PrP^{Sc})

Presently, the normal (cellular) prion protein (PrP^C) is believed to be the most important factor in the pathogenesis of TSE and its abnormal and partially

proteinase-resistant isoform, designated as PrP^{Sc}, might be the main or even sole factor responsible for TSE infectivity (DeArmond, Kretzschmar, & Prusiner, 2002). PrP^{C} proved to be relatively unspecific for the detection of CNS-based specified risk material (Weyandt, 2001; unpublished results). As an alternative analytical approach to the detection of SRM markers in meat products, it would be interesting to directly detect PrP^{Sc} as marker for TSE infectivity. While, in current BSE surveillance, several methods for the post-mortem detection of PrP^{Sc} in the CNS matrix are validated and officially approved (EC, 2001), it would be hard to test their applicability for the matrices of meat products in view of bio-security restrictions when producing the necessary standards with BSE-positive brains in conventional meat technology (kilogram range). This problem was solved by the development of a micro-technology for simulating meat production on a low scale (gram range), thus enabling the transfer of standard production into high-security laboratory (Lücker, Hardt, & Groschup, 2002). First results applying the Bio-Rad Platelia BSE purification and detection kits gave close linear relations between BSE-positive brain content in standards of emulsion-type micro-sausages which were linear in a range of up to 10% brain content and indicated detection limits as low as 0.25% brain. In follow-up studies the applicability of two novel immunoassays for the direct detection of PrP^{Sc} in meat products were tested: A sandwich immunoassay for PrP^{Sc} carried out following denaturation and concentration steps (Bio-Rad TeSeE test) and a two-sided immunoassay using two different monoclonal antibodies directed against two epitopes presented in a highly unfolded state of bovine PrP^{Sc} (Roboscreen Beta Prion BSE EIA Test Kit). Overall, these tests showed increased sensitivity, robustness and applicability (Grundmann, Kunath, Lücker, Hardt, & Groschup, 2005; Grundmann, Lücker, Hardt, & Groschup, 2004; unpublished results). This promising alternative analytical approach to directly detect markers of TSE infectivity in meat products, however, needs further studies for optimizing and validating the analytical procedure prior to its application in official food control.

BSE-Risk Markers from the Nucleic Acid Fraction

Molecular biological analytical tools are highly attractive for the detection of SRM in meat products in view of their extreme specificity and sensitivity. In principal, they also offer a species-specific characterization of detected CNS. This analytical approach is highly sophisticated as mRNA suitable for species-specific CNS detection has to be identified and concerns about mRNA stability in meat products and quantitative extractability as well as contamination aspects have to be met. However, first reports on the principal suitability of mRNA-based CNS detection were simultaneously published by two working groups as early as 2003 (Lange, Alter, Froeb, & Lücker, 2003; Seyboldt, John, Müffling, Nowak, & Wenzel, 2003).

Seyboldt and co-workers (2003) used a 168 bp CNS-specific GFAP mRNA target and reverse transcription PCR for tissue-specific CNS detection followed by RFLP in order to discern between bovine and non-bovine CNS species. The method was successfully applied along with a commercial GFAP-ELISA in one further study on CNS detection in retail liver sausages. The authors concluded that their PCR assay would be useful to characterize the CNS species in CNS-positive meat products as detected by immunological screening (Nowak, Muefffling, Kuefen, Ganseforth, & Seyboldt, 2005).

Lange and co-workers (2003) identified suitable target mRNAs for CNS. The selected primers for $GFAP_{87}$ and MBP_{51} facilitated the detection of CNS in raw sausages and non-heated and heated standards of emulsion-type sausages with defined addition of brain by reverse transcription PCR. Results indicated ample stability against meat technological influences such as storage, temperature and ripening. RT-PCR with $GFAP_{87}$ facilitated the detection of CNS without species specificity whereas MBP_{51} enabled the selective detection of the CNS of bovines, ovines and caprines but not CNS of porcines and poultry.

Gout, Valdivia, Mc Dowell, and Harris (2004) designed an assay using methylation-specific PCR (MSP). The authors isolated GFAP promoter fragments and identified key differences in the methylation patterns of certain CpG dinucleotides in the sequences from bovine and sheep brain and spinal cord and the corresponding skeletal muscle in order to specifically amplify the neuronal tissue-derived sequence and therefore identify the presence of CNS tissue.

Abdulmawjood, Schoenenbrücher, and Bülte (2005) report the development of a quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of CNS meat and meat products. This real-time RT-PCR assay used encoding gene sequences of bovine, ovine and caprine glial fibrillary acidic protein (GFAP) as markers. The mRNA assay facilitated the highly species-specific detection of bovine, ovine or caprine CNS tissues in meat and meat products. Bovine brain dilutions as low as 0.01% could be detected. The real-time RT-PCR assay was not affected by meat technological procedures, in particular heat treatment. In the first part of a national collaborative trial involving liquid extracts of meat products with varying brain content showed good reproducibility and detection limits as low as 0.1% (Abdulmawjood, Schönenbrücher, & Bülte, 2006). Further in-house studies on the validation and standardization of this real-time RT-PCR assay revealed high bovine tissue specificity and mRNA marker stability (Schönenbrücher, Abdulmawjood, Göbel, & Bülte, 2007). The authors concluded that their real-time RT-PCR assay appears to be a suitable tool in routine diagnostic testing for the illegal use of CNS tissue.

Even though inter-laboratory validation of extraction procedures and stability will have to be further addressed, the RT-PCR assays so far presented offer a very promising analytical approach for the detection of CNS with real potentials for species specificity and high sensitivity.

Conclusion

A panel of methods and markers for the detection of CNS-based SRM are currently available. Some have been validated and some are still under development. Overall, science has supplied official food control with ample means to compensate losses in safety against a potential human BSE exposition risk which might be introduced by lifting the ban on specified risk material. Astoundingly, combined research activities achieved a close analytical approximation to the extremely complex requirements of the legal definition of BSE risk material in case of CNS-based SRM.

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Chapter 20
GMO Detection

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Introduction

Modern agriculture and the food industry are under constant pressure to produce healthier, tastier and cheaper food, while at the same time maintaining and improving safety standards. Consequently, these industries are all the time demanding still better, more efficient genotypes of crop species and farm animals suited to a wide range of usages. Farmers, in particular, are calling for species that are more resistant to disease, that have improved adaptation to stress, and that facilitate simpler farming systems while also increasing yield and productivity. At the same time, scientists believe that such animal and crop varieties could provide a source of food for poor countries and, thereby, help to prevent, and ultimately eliminate, third-world malnutrition (Biotechnology Industry Organization, 2008; Monastra & Rossi, 2003; Herdt, 2006).

The genetic resources of plants and animals have been altered by centuries of careful selection. In the nineteenth century, Mendel's discoveries and his description of the laws of inheritance led to breeding improvements. Since his era, cross-breeding has been effectively applied to select the best performing progenies, and, more recently, complemented by other procedures, including plant tissue culture, induced mutagenesis, and wide hybridization (Gamborg & Sandøe, 2004; Solkner et al., 2008; Harbers, 2008; Collard & MacKill, 2008). With the discovery of the structure of DNA and demonstrations of the results of gene sequencing (Delseny et al., 1997; Bendixen, Hedegaard, & Horn, 2005; Snelling et al., 2007; Stein, 2007), the transfer of foreign genes into recipient cells, together with targeted cell transformation, became possible. Genetically modified organisms (GMOs) thus became reality (Hulse, 2004; Czaplicki, Ovesná, & de Vries, 2005).

In order to genetically modify an organism, a gene construct known as a transgene is inserted into the DNA of a host cell. If the new gene codes for a particular protein then, depending on the site at which the DNA integrates and

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other factors, this protein may be expressed in the host organism (procaryote, plant or animal). The transgene consists of a gene sequence coding for the protein of interest, a promoter sequence that regulates expression of the gene product, and a terminator of gene expression. Sometimes the purpose of modification is to 'knock out' a gene or to disrupt its function, rather than to add a new one (Pechan, 2005).

Today, genetically modified microorganisms (GMMs) are widely used in the large-scale production of various pharmaceuticals and food/feed additives (Kolodziejczyk & Fedec, 1999; Lupotto & Stile, 2007; Kamenarova et al., 2007; Ramessar, Sabalza, Capell, & Christou, 2008; Meyer, 2008; http://www.gmo-compass.org/eng/grocery_shopping/ingredients_additives/36.gm_microorganisms_taking_place_chemical_factories.html). Genetically modified (GM) plants are already used in the production of plant crops that are herbicide-tolerant and insect-resistant, such crops being subsequently used in animal feed (Pechan, 2005).

Although transgenic animals are still awaiting regulatory approval for wider commercial application, considerable progress has already been made in the development of GM farm and aquatic animals. Currently, the use of gene transfer in farm animals is focused on delivering benefits that include improved product quality and quantity; increased resistance to disease; the production of valuable proteins in their mammary glands or other organs; and the genetic modification of pigs for xenotransplantation (Wolf et al., 2000; Niemann, 2008). The development of aquatic GMOs is likely to offer similar benefits to commercial aquaculture (McLean & Devlin, 2000; Hallenan, McLean, & Fleming, 2007). Gene transfer experiments in this field have demonstrated several-fold increases in the growth rates of some fish species (Du et al., 1992; Rahman & MacLean, 1999), and increased tolerance to cold in others (Fletcher, Davies, & Hew, 1992). GM fishes are about to enter the industrial arena; in particular, fast-growing transgenic salmon is soon expected to appear on the market (Nam, Maclean, Hwang, & Kim, 2008). Thus, gene technology seems to have become established as a viable approach to the modification and improvement of traditional plants and farm animals.

Regardless of their commercial potential, the fact that GMOs and derived products are developed using new technology and may combine traits from various species has led to serious concerns about their impact on the environment and the threat they pose to human health (Van den Eede et al., 2004; Sayre & Seidler, 2005; Hug, 2008). Consequently, legislative measures have been introduced and corresponding regulatory and control systems established.

Basic Legislative Approaches to GMOs and GM Products

Its long history of usage means that traditional food is generally considered safe. Evaluation of the safety of such food is focused on the possible effects of specific chemicals, such as those used as food additives (Food and Agriculture

Organization, 2008; Hug, 2008; Kok, Keijer, Meter, & Kuiper, 2008; Marvier, 2008). However novel foods, without a significant history of consumption and derived from methods not previously used in food production, are subject to much stricter safety and risk assessment procedures.

Regulatory bodies require sound, unbiased advice on the safety of GM food and feed, and, therefore, take into account: (1) the findings of science-based safety evaluation and risk assessment systems aimed at objectively determining the benefits and risks of GM food (Singh, Ghai, Paul, & Jain, 2006; Bertoni & Marsan, 2005; Konig et al., 2004); (2) recommendations for the labeling of food and food ingredients produced by modern biotechnology; (3) assessments of the nutritional aspects of food derived from modern biotechnology; and (4) evidence of the detection of proteins and/or DNA sequences in GM foods. The basic principle underlying the risk analysis of a novel food is that it should be compared with its traditional counterpart so that their differences, together with their substantial equivalence, can be determined (Kok et al., 2008). Such a comparison involves evaluating the potential toxicity of a novel food product using bioinformatics (sequence homology with known toxins, new protein function) and, if applicable, animal feeding studies. Allergenicity, too, is examined by comparison with known allergens, special attention being paid to a new product derived from a known allergenic food. If necessary, the transgene expression level is also verified. Based on these fundamental principles, international guidelines and recommendations have been developed and generally recognized.

At the global level, food safety issues are covered by the *Codex Alimentarius* (De Leon-Garcia, 2007), which is maintained by the Codex Alimentarius Commission (CAC). The CAC was established in 1963 by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to develop food standards, guidelines and related texts, such as codes of practice, under the Joint FAO/WHO Food Standards Programme. The *Codex Alimentarius* itself is a collection of food standards compiled by the CAC, the main purpose of which is to protect consumer health and ensure fair trade practice in the food trade. *The Principles and Guidelines for foods derived from modern biotechnology*, the first guidelines adopted by the CAC to specifically deal with food products derived from modern biotechnology, thus far includes

- *The Codex Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* (CAC/GL 44-2003),
- *The Codex Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganism*s (CAC/GL 46-2003), and
- *The Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (CAC/GL 45-2003).

The demands of international trade have subsequently driven the formulation of two annexes to the guideline for recombinant-DNA (rDNA) plants (CAC/GL 45-200): (i) *Annex on Foods from rDNA Plants Modified for*

Nutritional or Health Benefits and (ii) *Low Level Presence Annex to Codex rDNA Plant Guideline* aim of which is to assist the safety assessment of rDNA crops not authorized in respective country, but permitted in the country of import.

With biotechnological procedures making rapid progress, and many transgenic animal products in the pipeline and awaiting approval to go to market, the *Guideline for Safety Assessment of Foods Derived from rDNA Animals*, which primarily addresses animals with heritable rDNA traits, was approved in 2008. While based on the principles anchored in the guideline for rDNA plants, it will also reflect the differences between plants and animals.

In the USA, the Federal Department of Agriculture (FDA) regulates the safety of food and feed products derived from modern biotechnology by using the standards and requirements that have traditionally applied under U.S. food law. Providing that they are not pesticides, substances added to food that do not meet the statutory definition of 'generally recognized as safe' are classified as food or color additives and must be pre-approved before they may be marketed. Since 1992, the U.S. Department of Agriculture, through the Cooperative State Research, Education and Extension Service (CSREES) and the Agricultural Research Service (ARS), has solicited research proposals in areas considered by the FDA, Environmental Protection Agency (EPA), CSREES, ARS, and Animal and Plant Health Inspection Service (APHIS) to be critical to the regulatory review and risk assessment process (Sayre & Seidler, 2005).

In Canada, novel foods are regulated under the Novel Foods Regulations, which define novel foods as products that have never been used as food, as foods that result from a process not previously used in the production of food, or as foods that, having undergone genetic modification, possess new traits. Novel foods are required to undergo a safety assessment prior to going to market. To date, more than 100 novel foods have been approved for sale in Canada.

The European approach to food safety reflects previous negative experiences with the introduction of new technologies and chemicals (e.g DDT, dioxins, etc.) and, as such, is strongly based on 'the precautionary principle' (Theofanis, 2004). Since April 2004 Regulation (EC) No 1829/2003 on GM food/feed has provided a single EU-wide procedure for the authorization of any novel food/feed product derived from a GMO and/or containing a GMO. Applications for the authorization of new GM products must be submitted to the European Food Safety Authority (EFSA), which then carries out a risk assessment and declares its opinion.

In the EU, GM food and feed must be labeled to protect the consumer's right to free and informed choice. Within the framework of the Biosafety Clearing House (Cartagena Protocol), the system of assigning a unique identifier to each GMO and GM product was established by the Organization for Economic Co-operation and Development (OECD). Each authorized GMO and GM product is given a unique identifier consisting of nine letters and/or numbers combined in a standard way; the first three characters corresponding to the organization submitting the application, the next five characters characterizing

Table 20.1 Examples of unique identifiers used to label GMOs

GA21 Maize	MON-00021-9	Monsanto
Mon863 Maize	MON-00863-5	Monsanto
1507 Maize	DAS-01507-1	Pioneer Hi-Bred, Dow Agrosciences, Mycogen Seeds
T25 Maize	ACS-ZM003-2	Bayer CropScience

the respective transformation event, and the trailing character serves as a verifier (e.g., see Table 20.1). The assignment of unique identifiers to, and the compulsory labeling of, GM products in the EU forms the basis for a legally mandated system of traceability. GMO admixtures of up to a certain proportion (0.9%) are excluded from EU labeling requirements in cases where the GM content in food/feed is unintentional and/or technically unavoidable, and where the GMO present has already been authorized in the EU.

The basic EU legislation (Arvanitoyannis, Choreftaki, & Tserkezou, 2005) covering GM food and feed consists namely of the following:

Regulation (EC) 1829/2003 on genetically modified food and feed.

Regulation (EC) 1830/2003 concerning the traceability and labelling of GMOs and the traceability of food and feed products produced from GMOs (amending Directive 2001/18/EC).

Commission Regulation (EC) 65/2004 establishing a system for the development and assignment of unique identifiers for GMOs.

Commission Regulation (EC) 641/2004 describing the detailed rules for the implementation of Regulation (EC) 1829/2003 with respect to applications for the authorization of new GM food and feed; the notification of existing products; and, the adventitious or technically unavoidable presence of GM material which has previously received a favorable risk evaluation.

Directive 2001/18/EC concerning the deliberate release of GMOs into the environment.

To ensure compliance with the regulations and requirements described above (Miraglia et al., 2004; Marabelli, 2005; Lezaun, 2006), the competent authorities require precise tools by which the presence of approved GM material and unapproved modifications can be detected.

Current Approaches to GMO Analysis

If an effective system of GMO traceability is to be established throughout the supply chain from seed producer to final consumer, reliable and efficient methods of GMO detection are essential (Deisingh & Badrie, 2005; Žel, Cankar, Ravnikar, Camloh, & Gruden, 2006; Starbird, 2007).

Methods suited for metabolic profiling may provide one way of identifying GMOs according to their new properties. Oil content and composition,

modified starches or fibers can all be detected by HPLC, MALDI TOF, NMR, or nanotechnology-based techniques (Hazebroek, 2000; Daniell, Ruiz, & Dhingra, 2005; Wang, & Welti, 2006; Brown, Kruppa, & Dasseux, 2005; Sauter, Lauer, & Fritsch, 1991; Lechner & Rieder, 2007; Metz et al., 2007; Babu et al., 2008). However, although these techniques are highly precise and efficient in detecting various compounds, they have thus far not been adopted for GMO screening. Instead, the inserted DNAs and corresponding proteins are typically used as analytical targets (Ermolli et al., 2006; Markoulatos et al.; 2004), with enzyme-linked immunosorbent assay (ELISA) being widely used for the identification of known proteins. The potential of this technique was confirmed during routine analysis for the presence of transgenic Roundup Ready soya and LL601 rice (Kasama et al., 2005). Consequently, other ELISA protein detection assays are currently under development. However, as genetic modification is caused by the insertion of a specific transgene into a target organism, DNA sequences are generally used as target analytes in GMO analysis (Anklam, Gadani, Heinze, Pijnenburg, & Van Den Eede, 2002; Ahmed, 2002; García-Cañas, Cifuentes, & Gonzalez, 2004; García-Cañas, González, & Cifuentes, 2004; Hernandez, Rodriguez-Lazaro, & Ferrando, 2005). DNA is more stable than protein so that even highly degraded DNA may be identified. The analytical procedure itself involves the following sequence of steps: sampling; sample processing; analyte isolation; and, interpretation of the analytical results obtained. The final analytical results are dependent on the selection of the most appropriate method and its proper execution and are only valid if validated procedures are used throughout (Holst-Jensen & Berdal, 2004).

Sampling of Food/Feed Products

Sampling is a critical step in GMO detection. In order to be confident that the results of GMO analysis are accurate (i.e., that GMOs and derived products have been detected where they should be), sampling and, subsequently, the analytical procedure itself must be carried out using sound scientific and statistical protocols (Beismann, Finck, & Seitz, 2007; Degrieck, Silva, Van Bockstaele, & De Loose, 2005; Brera et al., 2005; Anklam et al., 2002; Kobilinsky & Bertheau, 2005). For example, in analyzing a sample containing 3 000 particles (e.g., kernels), it is necessary to be aware that the statistical probability of detecting 1% GMO contamination in such a sample is $p \leq 0.05$. As the more particles there are in a sample the higher the probability of detecting the presence of GMOs, test samples should be prepared in such a way as to reflect the anticipated GMO content of a tested food/feed product.

With the appropriate procedure it is a relatively simple task to verify the identity of declared GMOs. However, detection is primarily carried out to identify the presence of GMOs in products declared as non GM as well as to

identify contamination by other GM materials, and to verify that GM products have been correctly labelled and handled. When planning the sampling of a big consignment containing products from multiple overseas suppliers, it is necessary to take into account the fact that GM particles are randomly distributed throughout. This is one of the reasons for a certain degree of lot heterogeneity (Paoletti, Donatelli, Kay, & Van Den Eede, 2003; Paoletti et al., 2006) and explains why, in some parts of the consignment, GM contamination hot spots may be identified. Soybean shipments sampled and analyzed at point of entry to the EU have confirmed this (Paoletti et al., 2006). For large consignments, it is advisable, in accordance with the approved procedure, to separately analyze several increments (sub-samples) during unloading. In the EU precise guidelines for the sampling of bulk agricultural commodities are already in place (Kay & Paoletti, 2001). A good knowledge of shipment origin is also important.

The EU sampling protocol (Commission recommendation 2004/787/EC) is a two-step procedure that enables the user to obtain estimates of GMO presence levels, together with their associated uncertainty expressed as standard deviation (SD). It recommends that the sampling of bulk commodities (e.g., grains, oilseeds) should be performed in accordance with the general principles and methods described in ISO standards 6644 and 13690, the number of increments recommended in each case corresponding to lot size (see Table 20.2).

The protocol recommends that the sampling of pre-packaged food/feed products should be carried out in accordance with the procedures described in ISO 2859.

With respect to the detection of low-level contaminations, it is illustrative to consider the EU sampling protocol (included in EU Recommendation 2004/787/EC) specifically developed for the detection of traces of LL601 GM rice in conventional rice. This protocol, developed on a statistical basis and recommending a laboratory sample size of 2.5 kg in duplicate, has already been used in the successful detection of LL601 rice (Freese et al., 2007), thereby showing that such procedures can be used in sampling lots for other types of GM contamination.

Regarding other types of GM material, seeds, for example, should be sampled in accordance with the International Rules for Seed Testing (ISTA), and samples of plant-propagating materials should be obtained in accordance with international standards so far as they exist (Degrieck et al., 2005; Emslie, Whaites, Griffiths, & Murby, 2007; Remund, Dixon, Wright, & Holden, 2001).

Table 20.2 EU recommendations for sizes of laboratory samples and number of increments (sub samples)

Lot size in tones	Size of the bulk sample in kg	Number of incremental Samples
≤ 50	5	10
100	10	20
250	25	50
≥ 500	50	100

Preparation of an Analytical Sample (Test Portion)

Once a laboratory sample has been prepared, it is sent to an appropriate testing facility and further processed. The sample material is carefully mixed and divided, and, in the case of dry material, ground or milled to achieve particles of, as far as possible, identical size. At this stage measures must be taken to prevent secondary contamination of the sample. Strategies for the homogenization of the laboratory sample, the reduction of the laboratory sample to a test sample, and the preparation of test portions are described in full in EN ISO 24276:2002 and EN ISO 21571:2002. The analysis itself is usually run in duplicate or triplicate.

DNA Isolation

Various different methods exist for DNA isolation, some of them being described in international standards and others being offered by commercial companies (Ferrari et al., 2007; Waiblinger, Ernst, Graf, & Pietsch, 2005; García-Cañas et al., 2004; Jerman et al., 2005; Smith & Maxwell, 2007). The method selected must be fit for purpose (Bonfini, Heinze, Kay, & Van den Eede, 2001), and the DNA used amplifiable and free of PCR (polymerase chain reaction) inhibitors/enhancers. DNA quality can be tested electrophoretically, spectrophotometrically, or fluorometrically. High molecular DNA may be isolated from, for example, fresh plant and animal tissues. However, partially or highly degraded DNA is usually obtained from processed matrices because during food processing DNA integrity is affected by several factors, including the higher pressures and/or elevated temperatures used during the crushing, milling, pasteurization, and/or baking processes, any one of which leads to DNA fragmentation (Moreano, Busch, & Engel, 2005). Food additives, too, may impact DNA stability, and further down the line, impact DNA analysis, if they are not properly removed during DNA isolation and purification.

Briefly, DNA isolation requires the lysis of intact cells in food products, an appropriate buffer containing lytic agents (e.g., cetyl trimethyl ammonium bromide – CTAB or sodium dodecyl sulphate – SDS), proteolytic enzymes, RNases, and DNAse inhibitors. After the cell debris and proteins have been removed by either precipitation or extraction in organic solvents, the DNA is further purified by either precipitation in ethanol or gel filtration.

With requirements differing for plant leaf tissue, kernels, blood, unprocessed food/feed and processed food/feed, each DNA isolation procedure must be carefully tailored and validated for individual matrices. The published protocols permit DNA isolation from various sources including meat, meat-based products, plant-based products, etc. (Buntjer, Lenstra, & Nel, 1995; Brod & Arisi, 2005; Di Pinto et al., 2007; Chapela et al., 2007; Dos Santos Ferrari et al., 2007). Phenol–chloroform-based methods are recommended for protein-rich

matrices, such as products containing meat. Specific modifications for these methods are available for smoked meat products containing bacterial starting cultures (Straub, Hertel, & Hammes, 1999; Federal Health Office, 2001). A CTAB-based method is usually appropriate for fresh plant tissue, while a guanidinium thiocyanate–phenol–chloroform-based method normally works well for both fatty products and highly processed food products (ISO 21571:2005 – *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic acid extraction*).

A basic estimate of DNA quality and quantity can be determined using gel electrophoresis, fluorometry or UV spectrophotometry. Gel electrophoresis is used when concentrations of a known standard can be compared with the sample. However, when DNA is degraded it becomes difficult to make such a comparison. Fluorometry and UV spectrophotometry are more precise and, enable a rough estimate of DNA purity to be obtained. Providing that the DNA is amplifiable, amplifiability is confirmed by the amplification of a species-specific sequence. Sets of PCR assays are already available for several species. After amplification, the isolated DNA can be further analyzed.

GMO Identification

PCR using Taq polymerase and sequence-specific primers allows the amplification (multiplication) of a target sequence. In the case of GMOs, short amplicons are normally used for the detection of even highly fragmented DNA. The amplification products are further separated by gel electrophoresis and then visualized under UV light or directly detected by real-time PCR. It is generally advisable to first screen for genetic elements, such as promoters and terminators. This screening step typically targets elements found in multiple GM events (ISO 21569:2005 – *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Qualitative nucleic acid-based methods*). For example, the combination of four genetic elements (35S CaMV and T-NOS promoters; 35S CaMV and NOS terminators) enables identification of almost all transgenic maize events (both approved and unapproved in the EU). In accordance with EU legislation, once a sample has been screened as GM-positive, the specific event must be identified.

To facilitate international trade and traceability, and to ensure compliance with regulations based on the *Codex Alimentarius* consensus, FAO is in the process of setting up a global database that will contain information on all approved events wherever they occur in the world. This database will include a description of the respective event, the safety/risk assessment conducted for it, the method used to detect it, and the availability of any control material for it. Useful information about this initiative can be found on the websites of the United States Department of Agriculture (USDA – http://www.usda.gov),

EPA (www.epa.gov), BIO (http://www.bio.org), and RIKILT (http://www.rikilt.wur.nl) that is a part of Wacheningen Research Center in the Netherlands.

If an approved event is detected in the EU or any country requiring the labelling of GMO products, quantification is the next step.

GMO Quantification

In certain parts of the world (EU member states, Japan) the quantification of GMOs is required to fulfill legislative requirements. Real-time PCR is the technique of choice for the quantification of nucleic acid, the successful quantification of which is critically dependent on the quality of the DNA in the sample being analyzed (Giovannini and Concillo, 2002; Hernandez et al., 2005). Quantification involves making a relative comparison between the content of a species-specific (comparator) sequence and that of a transgenic one. Although GMO quantification may appear to be a routine procedure, it is necessary to take into account several factors that can complicate the analysis (Cankar, Štebih, Dreo, Žel, & Gruden, 2006). For example, the DNA from a processed matrix may be damaged and so bias the analytical method or method performance may vary for comparator and transgene (La Paz et al., 2007). Measurement uncertainty, therefore, must be considered in such analyses, especially where precise contamination thresholds are laid down by the relevant regulations being applied. Intermediate precision, a measure between repeatability and reproducibility, best reflects the actual situation in laboratories dealing with quantitative aspects of molecular biology methods (Žel, Gruden, Cankar, Štebih, & Blejec, 2007).

Full details of the GMO quantification procedure may be found in the appropriate ISO standards (ISO 21570:2005 *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid-based methods;* ISO 24276:2006 *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – General requirements and definitions*), while the use of real-time PCR for GMO quantification has been described and discussed by various authors (Cankar et al., 2006; Bonfini et al., 2001; Toyota et al., 2006).

In the EU the methods used to both identify and quantify transgenic events are validated by the Community Reference Laboratory (CRL) at the EU Joint Research Centre (JRC) in Ispra, Italy, and are available at http://gmo-crl.jrc.it/statusofdoss.htm.

Identification of Unapproved GMOs

The methods outlined above enable the efficient identification and, as required, quantification of approved GM events. Global expansion in the development and cultivation of GMOs has naturally led to an increase in approved GM

events and, in some cases, to the asynchronous approval of certain GM events in different countries. For example, GM events in canola, corn, soybean, cotton, flax, papaya, potato, squash, sugar beet and tomato are among those to have received regulatory approval in the USA and Canada, while far fewer GM events have been approved in the EU. Some unapproved GM events have appeared in countries where they were not previously approved. Unapproved GM events are illegal wherever they occur and, in the EU at least, are treated as emergency cases (e.g., T25 canola, LL 601 rice, 1300 corn, Bt 63 rice).

The increasing number of GMOs appearing on the market, together with the growing number of cases involving their unapproved adventitious presence, is impacting both the domestic and international trade of many countries. This situation challenges the scientific community to rapidly develop high-throughput detection and quantification methods; a challenge that is complicated by the unavailability, in many cases, of certified reference material, by the lack of DNA sequence information regarding the design of event-specific primers, and by the sheer number of individual events that may be present and require testing for (Demeke, Perry, & Scowcroft, 2006; Moreano, 2007). On the bright side, a number of promising approaches are currently being developed and considered for GMO analysis.

One such approach involves the application of the ligation-dependent probe amplification (LPA) technique for the simultaneous detection of multiple GMO target sequences. This new strategy results in an open, modular system, which, because it can incorporate multiple probes, broadens the range of detected sequences and, thereby, helps meet the challenges posed by the steadily increasing number of GMOs (Moreano, Ehlert, Busch, & Engel, 2006).

DNA arrays/chips represent another potentially effective approach that might enable the detection of hundreds of globally approved GM events in either real-time PCR formats or low-density DNA arrays (Roy & Sen, 2006; Bai et al., 2007). Leimanis et al. (2006) described the use of capture probes on slides hybridized with the products of multiplex PCR for the simultaneous detection of tens of GMOs. Where Leimanis et al. (2006) silver stained the products of hybridization, Xu et al. (2007) used the more expensive technique of fluorescent labeling for the analyzed DNA. Scientists, generally, are looking for ways to bypass the amplification step, but the high DNA concentration required for whole genome hybridization seems to be a real obstacle, thereby currently limiting the use of DNA arrays. Bypassing this step has been shown to be theoretically possible at the experimental level (Nesvold, Kristoffersen, Holst-Jensen, & Berdal, 2005). Promisingly, Chen et al. (2006) suggested using Klenow fragment to directly label DNA analytes without pre-amplification. However, such an approach requires testing on a massive scale. It is to be hoped that, complemented by bioinformatics tools, it may eventually prove possible to successfully use both DNA arrays/chips and LPA for the simultaneous detection of multiple approved and unapproved GM events.

In the EU the potential health risks posed by unapproved GMOs are considered to justify the high cost of undertaking analysis for their detection.

The European Network of GMO Laboratories (ENGL) will shortly publish guidelines on the detection of unknown GMOs based on the results of work it is carrying out in partnership with the CRL and the Co-Extra EU project (http://www.coextra.eu).

Quality Control

The GMO analysis carried out in control laboratories must meet the criteria set out in the Good Laboratory Practice (GLP) regulations and by the system of accreditation under which the individual laboratory operates. Both GLP and ISO (International Standards Organization) accreditation standards help to ensure that analytical results are reliable. Accreditation is a suitable system for the standardization of procedures among individual laboratories, quality management being its basic principle. Each accredited laboratory must have an established quality system, detailing its quality procedures and system of continuous improvement. Žel et al. (2006) described the key elements that, in accordance with ISO/IEC 17025: 2005, must be in place for the accreditation of molecular biology methods used in the detection of GMOs. Furthermore, if reliable and comparable results are to be obtained, such methods, once accredited, must be performed in a uniform way by all laboratories using them.

To assess the results obtained by PCR-based methods, validation data describing the sensitivity, specificity, precision (repeatability, reproducibility, robustness), and accuracy (reliability) of such methods are required. The performance criteria for these methods have been described in detail (Bertheau, Diolez, Kobilinsky, & Magin, 2002, http://biotech.jrc.it/validation.htm] and should be verified by trained personnel in each laboratory. To ensure measurement traceability, control laboratories should use reference materials, the best of which are currently produced by the Joint Research Center – Institute for Reference Materials and Measurements (JRC-IRMM) in Geel, Belgium. Additionally, the CRL produces verified plasmid controls for EU control laboratories.

To ensure the credibility of published results a system of internal and external controls is used. To identify errors in the analytical procedures being performed by participating laboratories, and thereby monitor the validity of GMO analysis overall, a vitally important system of proficiency testing has been organized by regulatory bodies in different regions. These bodies include the Institute of Food Research (IFR, UK), the Central Science Laboratory (CSL – GeMMA Scheme, UK), and the USDA/GIPSA (US Grain Inspection, Packers and Stockyards Administration) Proficiency Program. Such testing helps to maintain a system of objective standards that individual control laboratories must meet if they wish to compare their analytical results with those of others.

Conclusions

Today, both international and national regulatory frameworks exist for the handling, traceability, and detection of GMOs. Protein-based and DNA-based methods have already been approved as fit for the purpose of detecting and quantifying transgenic events. However, with the ever-increasing number of GMOs available on the market, the immediate challenge is to develop and verify new approaches that enable multiple approved GM events to be detected simultaneously with the presence of unapproved GMOs. The development of such methods constitutes the main challenge for the future, and, therefore, the primary focus of current research.

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Chapter 21
Principles of Predictive Modeling

Marie Laure Delignette-Muller

Introduction

Mathematical models were first used in food microbiology in the early 20th century to describe the thermal destruction of pathogens in food, but the concept of predictive microbiology really emerged in the 1980 s. This concept was first developed and extensively discussed by McMeekin and his colleagues at the University of Tasmania (Ratkowsky, Olley, McMeekin, & Ball, 1982; McMeekin, Olley, Ross, & Ratkowsky, 1993; McMeekin, Olley, Ratkowsky, & Ross, 2002). Now predictive microbiology or predictive modeling in foods may be considered as a subdiscipline of food microbiology, with its international meetings (5th conference on "Predictive Modelling in Foods" in 2007) gathering a scientific community from all over the world.

In predictive microbiology, mathematical models are used to predict the behavior of a microbial population in food from a detailed knowledge of the type of microorganism and of its environmental conditions (intrinsic and extrinsic factors characterizing the food and its environment). Microorganisms of interest in predictive microbiology are both foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Clostridium botulinum* and spoilage microorganisms such as *Pseudomonas* spp. and *Enterobacter* spp. Food products of interest are those that may be naturally contaminated by pathogens or spoilage microorganisms or those contaminated during process, for which process conditions do not ensure a complete destruction of microorganisms. Meat and processed meat obviously fall under this category. Food products may be characterized by intrinsic factors such as pH and water activity (a_w) and extrinsic factors such as temperature and storage atmosphere. Intrinsic and extrinsic factors directly affect the microbial behavior

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in food. In predictive microbiology, models are developed to describe the effect of these factors on microbial kinetics. Models are often developed through two steps. In the first step primary models are developed to describe microbial growth or survival kinetics in a constant environment. These models are characterized by parameters such as maximum specific growth rate μ_{max} and lag time λ for growth kinetics or decimal reduction time D for inactivation kinetics. In the second step, secondary models are developed to describe the effect of environmental intrinsic (pH, a_w, etc.) and extrinsic factors (temperature, atmosphere, etc.) on parameters of primary models (μ_{max}, λ, D, etc.). Primary and secondary models may then be used together to predict the microbial behavior in food in constant or changing environmental conditions. In this chapter we will review the more common primary and secondary models and discuss their applications in food microbiology and especially for safety of meat.

Primary Models

Despite the development of novel methods, viable count determinations on agar plates are still commonly used in food microbiology, and this method remains the method of reference to acquire data for developing primary models. It enables the enumeration of even very small numbers of microbial cells and may be used not only on liquid media but also directly on food samples. But as it is a time-consuming and laborious method, the construction of models from such data remains a heavy task. Moreover, the level of error in viable count data should be kept in mind while developing predictive models. Primary models aim to describe the microbial observed kinetics the more accurately possible with a minimum number of parameters. Models should have no more parameters than are required to describe the kinetics as an excessive number of parameters can lead a model to describe the error in the data. This concept is named the parsimony of models (Ratkowsky, 1990). Another quality expected is the interpretability of the parameters of a model (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990). Parameters of primary models should have a clear biological interpretability, such as the maximum specific growth rate μ_{max}, the lag time λ, and the initial cell concentration N_0.

Growth Models

Microbial growth kinetics are commonly described in at least three stages: the lag phase, the exponential phase, and the stationary phase (Fig. 21.1). A multiplication of the microbial cells is observable only in the exponential phase. During this phase the time taken by the microbial population to double is supposed constant, equal to the generation time t_g. This parameter is directly linked to the maximum value μ_{max} of the specific growth rate dN/Ndt, with N the cell concentration, by the relation $t_g = \ln(2)/\mu_{max}$. The maximum specific

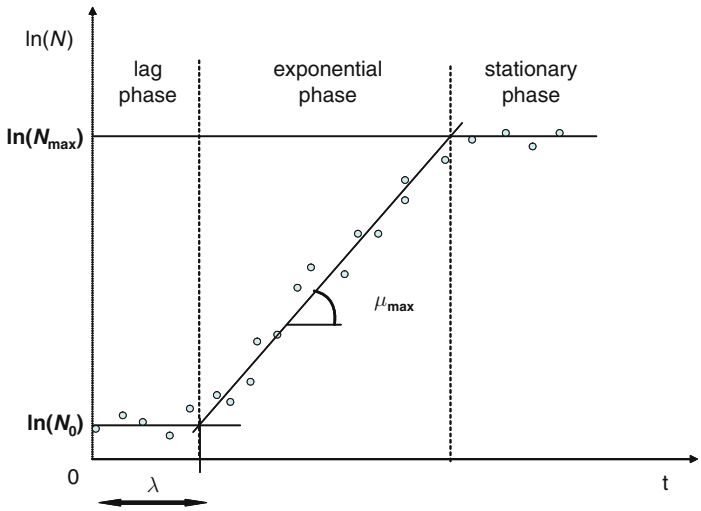

Fig. 21.1 Phases of a microbial growth curve and classical growth parameters

growth rate $\mu_{\max}$ is also the slope of the kinetics in the exponential phase if the natural logarithm of N ($\ln(N)$) is represented as a function of time t (Fig. 21.1), as a simple formulation of the growth model in the exponential phase is

$$\frac{dN}{Ndt} = \frac{d\ln(N)}{dt} = \mu_{\max} \tag{21.1}$$

Traditionally, $\mu_{\max}$ and the lag time λ have been determined by drawing a line through the exponential phase of the curve and defining λ as the time when this line intersects an horizontal tangent to the curve at initial time t_0 (Fig. 21.1). But this method may be quite subjective, especially where there are a limited number of data points or where these points are not strictly lined. Many authors have proposed primary models to fit the whole growth kinetics and thus improve the estimation of growth parameters. We will not detail all the proposed models which can be found in various reviews (Zwietering et al., 1990; van Gerwen & Zwietering, 1998; Baty & Delignette-Muller, 2004). We will focus on three popular models: the Baranyi model proposed by Baranyi and Roberts (1994), the three-phase linear model proposed by Buchanan, Whiting, and Damert (1997), and the modified Gompertz model first introduced by Gibson, Bratchell, and Roberts (1988) and reparameterized by Zwietering et al. (1990). The Baranyi and the three-phase linear model may both be presented as an extension of the exponential model (Eq. 21.1) in order to encompass lag and stationary phases. They can be written from one unified equation (Eq. 21.2), just

differing by their definition of the adjustment and inhibition functions, respectively, $\alpha(t)$ and $f(N)$.

$$\frac{dN}{Ndt} = \frac{d\ln(N)}{dt} = \mu_{\max}\alpha(t)f(N) \tag{21.2}$$

In the Baranyi model, the adjustment function describes the adjustment of the culture to its new environment during lag phase by

$$\alpha(t) = \frac{q(t)}{1 + q(t)} \tag{21.3}$$

where $q(t)$ represents the physiological state of the microbial population. This physiological state is supposed to be proportional to the concentration of a nonidentified critical substance and to follow the first-order kinetics

$$\frac{dq}{qdt} = \mu_{\max} \text{ with } q(0) = q_0 = \frac{1}{\exp(\mu_{\max}\lambda) - 1} \tag{21.4}$$

with $\mu_{\max}$and λ representing the maximum specific growth rate and the lag time, respectively.

In the Baranyi model, the classic logistic function is used as an inhibition function

$$f(N) = 1 - \frac{N}{N_{\max}} \tag{21.5}$$

where $N_{\max}$ is the maximum cell concentration reached in the stationary phase. The Baranyi model can be written in an analytical form usable to describe kinetics obtained in a constant environment (Eq. 21.6):

$$\log_{10}(N) = \log_{10}(N_{\max}) + \log_{10}\left(\frac{-1 + \exp(\mu_{\max}\lambda) + \exp(\mu_{\max}t)}{\exp(\mu_{\max}t) - 1 + \exp(\mu_{\max}\lambda + \ln(N_{\max}) - \ln(N_0))}\right) \tag{21.6}$$

To describe microbial kinetics in changing conditions, implying for example a changing value of the parameter $\mu_{\max}$ due to variations of the temperature with time, the differential form of the Baranyi model (Eq. 21.2 with Eqs. 21.3, 21.4, and 21.5) should be used.

The three-phase linear model is also based on Eq. 21.2, but with rather simple definitions of the adjustment and inhibition functions:

$$\alpha(t) = \begin{cases} 0\ , & t \leq \lambda \\ 1\ , & t \leq \lambda \end{cases} \tag{21.7}$$

$$f(N) = \begin{cases} 1\ , & N < N_{\max} \\ 0\ , & N \geq N_{\max} \end{cases} \tag{21.8}$$

As the Baranyi model, the three-phase linear model can be written in an analytical form usable to describe kinetics obtained in a constant environment (Eq. 21.9):

$$\log_{10}(N) = \begin{cases} \log_{10}(N_0)(t \leq \lambda) \\ \min\left(\log_{10}(N_0) + \dfrac{\mu_{\max}(t-\lambda)}{\ln(10)}; \log_{10}(N_{\max})\right)(t > \lambda) \end{cases} \tag{21.9}$$

The modified Gompertz model is a completely empirical one and has only an analytical formulation:

$$\log_{10}(N) = \log_{10}(N_0) + \log_{10}\left(\frac{N_{\max}}{N_0}\right) \exp\left(-\exp\left(\frac{\mu_{\max}\exp(1)}{\ln(N_{\max}) - \ln(N_0)}(\lambda - t) + 1\right)\right) \tag{21.10}$$

This model was first proposed by Gibson et al. (1988) to describe microbial growth curves and reparameterized by Zwietering et al. (1990) in order to define it with classical growth parameters N_0, $N_{\max}$, $\mu_{\max}$, and λ. But the biological interpretability of its parameters is not so obvious since Eq. 21.10 does not describe a value of N equal to N_0 at time $t = 0$ and since the model is a logistic one with $\mu_{\max}$ representing the slope of the tangent of the curve at the inflection point and not the constant slope of the curve during the exponential phase as expected.

When these primary models are used to estimate growth parameter from kinetics data, they are generally fitted by nonlinear regression to data points transformed in logarithm (on $\log_{10}(N)$ or $\ln(N)$), using an additive Gaussian error model. This can be done using any statistical software providing a non-linear regression function or using specific software. For example, DMFit is an Excel add-in freely provided by the Institute of Food research on its web site (http://www.ifr.ac.uk/safety/DMFit/default.html) that enables the fit of growth kinetics using the Baranyi model or the three-phase linear model or simplified versions of both the models, assuming, for example, that there is no lag phase or that stationary phase was not reached. In the R package for nonlinear regression diagnostics (nlstools, 2007), the same models are proposed with the modified Gompertz one in addition. This package may be directly installed from the free R language (R Development Core Team, 2007) or downloaded from the web site of R foundation for Statistical Computing (http://lib.stat.cmu.edu/R/CRAN/) and used with the R language. Figure 21.2

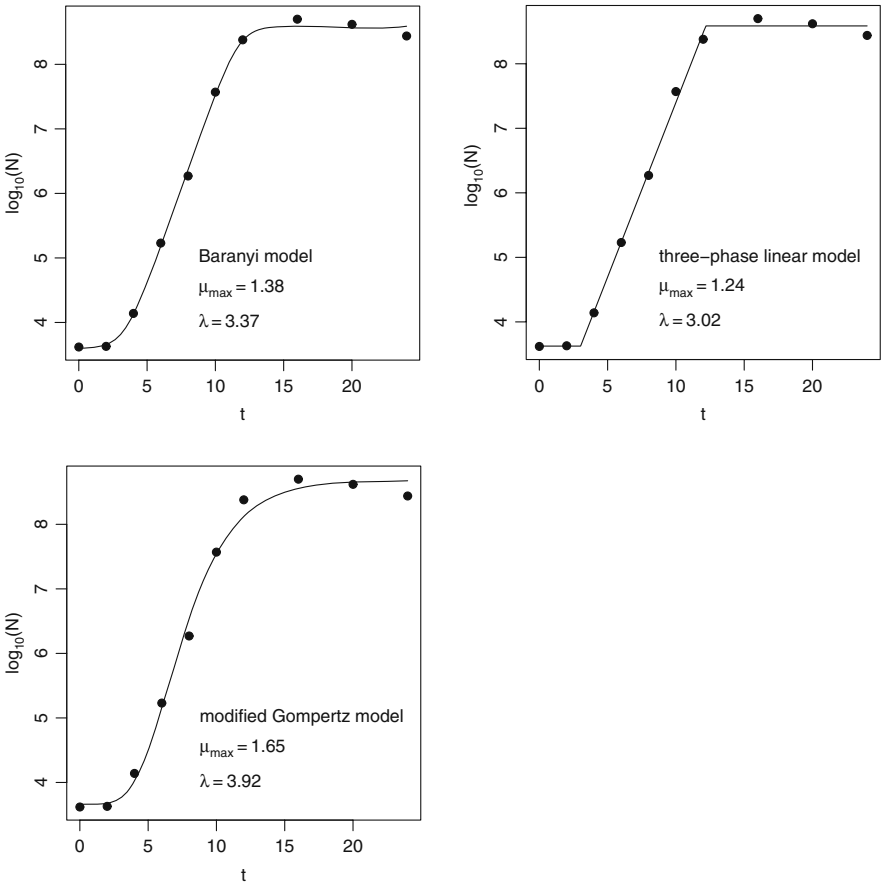

Fig. 21.2 Fitting of three classical primary growth models on the same growth data set

shows the three models fitted on the same growth data set using functions provided by nlstools.

When these models are compared on different data sets, none of them systematically gives a better fitting for all the data sets. They all seem to fit data sets quite well even if they do not give exactly the same estimations of growth parameters (McKellar & Lu, 2004). The modified Gompertz model, due to its logistic form, is known to overestimate the maximum specific growth rate in comparison to other models and tends to give more imprecise estimations of growth parameters for kinetics with few data points (Baty & Delignette-Muller, 2004). Moreover, the Baranyi model and the three-phase linear model are more flexible, with simplified versions for the cases where there is no lag phase or where the stationary phase is not reached at the end of the kinetics, and they both have a differential formulation which permits their use in changing environmental conditions.

Survival Models

The classical model still often used to describe survival kinetics is the log-linear model:

$$\frac{dN}{Ndt} = \frac{d\ln(N)}{dt} = -k_{\max} \tag{21.11}$$

where N is the cell concentration and $k_{\max}$ the maximum specific inactivation rate. The second parameter $k_{\max}$ is directly linked to the classical decimal reduction time D by $D = \ln(10)/k_{\max}$, often named the D-value. But microbial survival kinetics, whether due to a thermal or a nonthermal food processing, may present various forms differing from the linear one. Geeraerd, Valdramidis, and Van Impe (2005) described nine typical forms characterized by a concavity or convexity and the possible presence of a tail and/or a shoulder and reviewed the various primary models proposed by different authors to describe all the possible forms (Geeraerd, Herremans, & Van Impe, 2000). Some of these models are adaptations of the Baranyi growth model, considering a survival curve as a mirror image of a growth curve. The model proposed by Geeraerd et al. (2000) is of this type and is classically parameterized by N_0 the initial cell concentration, N_{res} the residual cell concentration at the end of the kinetics, $k_{\max}$ the maximum specific inactivation rate, and S_l the shoulder (in time):

$$\frac{dN}{Ndt} = \frac{d\ln(N)}{dt} = -k_{\max}\alpha(t)f(N) \tag{21.12}$$

with

$$\alpha(t) = \frac{1}{1 + C_c(t)}, \text{ with } \frac{dC_c}{C_c dt} = -k_{\max}, \text{ and } C_c(0) = \exp(k_{\max}S_l) - 1 \tag{21.13}$$

and

$$f(N) = 1 - \frac{N_{res}}{N} \tag{21.14}$$

Using the same analogy between a survival curve and a growth curve, it is also easy to define a three-phase linear model characterized by the same four parameters by Eq. 21.12 with Eqs. 21.15 and 21.16:

$$\alpha(t) = \begin{cases} 0, & t \leq S_l \\ 1, & t \leq S_l \end{cases} \tag{21.15}$$

$$f(N) = \begin{cases} 1, & N > N_{\text{res}} \\ 0, & N \leq N_{\text{res}} \end{cases} \tag{21.16}$$

Some authors also use models derived from the Weibull model first proposed by Peleg and Cole (1998) and adapted by other authors (Mafart, Couvert, Gaillard, & Leguérinel, 2002; Albert & Mafart, 2005). The initial Weibull model is based on the assumption that the time required to cause the death of one cell is variable in the microbial population and follows a Weibull distribution. The last model proposed by Albert et al. is parameterized by four parameters: N_0 the initial cell concentration, N_{res} the residual cell concentration at the end of the kinetics, δ the time of the first decimal reduction, a parameter close to the classical D-value, and p a shape parameter, characterizing the curve convexity or concavity. This model can be written in a similar form as the two others, by Eqs. 21.12, 21.14 and 21.17:

$$\alpha(t) = -\frac{\ln(10)}{\delta^p} p t^{p-1} \tag{21.17}$$

As growth models, survival models are generally fitted by nonlinear regression to data points transformed in logarithm (on $\log_{10}(N)$ or $\ln(N)$), using an additive Gaussian error model. Geeraerd et al. (2005) developed an Excel add-in named Ginafit, which is freely provided by the University of Leuven (http://cit.kuleuven.be/biotec/). It enables the fit of survival kinetics using nine different models, among which the Geeraerd model and Albert model previously presented their simplified versions assuming for example that there is no shoulder or no tail. In the R package nlstools, the fit of eight models, the three models previously described, and their simplified versions are proposed. Figure 21.3 shows these three models fitted on the same survival data set using functions provided by nlstools.

The Geeraerd model and the three-phase linear model give generally close estimations of the four classical parameters N_0, N_{res}, k_{max} and S_l. The simple interpretability of their parameters is an obvious quality of these models. Nevertheless, they do not enable the fit of all the survival curves. In fact, the form of survival curves is very variable and dependent on the type of food processing. In this context, the Weibull-type models (Mafart et al., 2002; Albert & Mafart, 2005) are sometimes preferred in predictive modeling works for their better fit to the data (Fernández, López, Bernardo, Condón, & Raso, 2007; Janssen et al., 2007; Leguérinel et al., 2007).

Secondary Models

Secondary models are developed to describe the effect of environmental factors on behavior of microorganisms. They are generally developed for one microbial species, from experiments made on one or various strains of the species. Most of them describe the effect of environmental parameters directly on the primary growth or survival parameters. In order to develop such models, it is thus necessary to measure many growth or survival kinetics in different

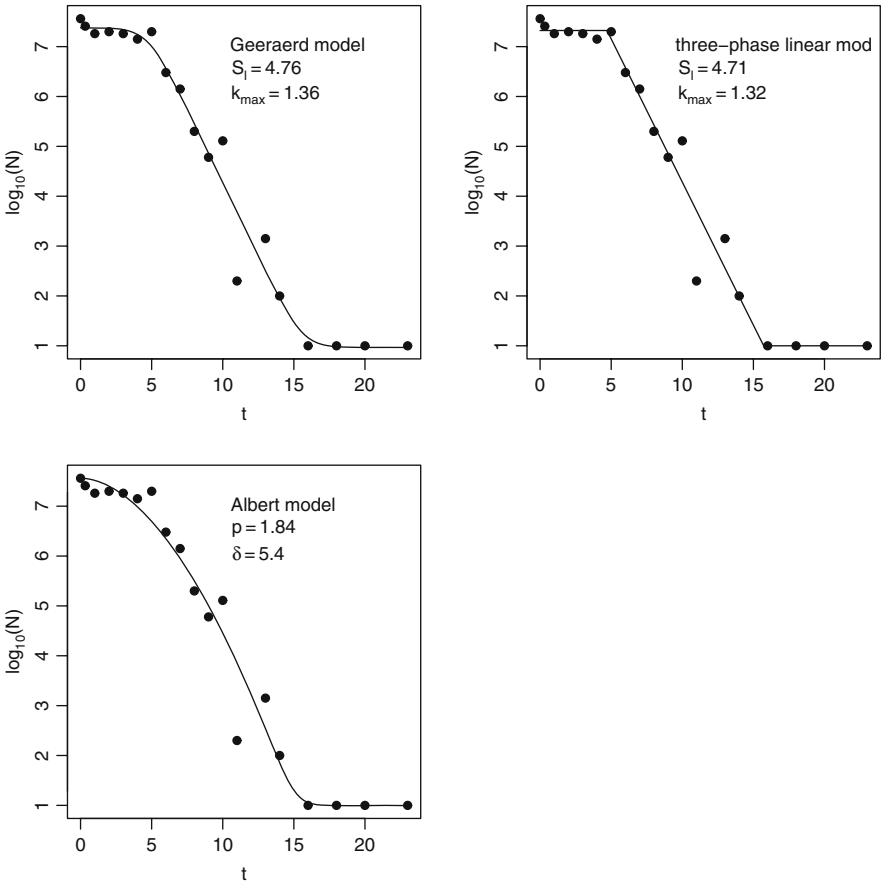

Fig. 21.3 Fitting of three classical primary survival models on the same survival data set

environmental conditions characterized by one or more factors. The experimental design is of great importance at this stage (van Boekel & Zwietering, 2007). After data collection, it is necessary to fit a primary model on each of those kinetics, then to fit a secondary model to the estimated values of these parameters as a function of the environmental factors. A global fit of the whole data set is also possible and even preferable from a statistical point of view, but needs a greater statistical skill. As primary models, secondary models should be parsimonious, with no more parameters than are required to describe the data (Baranyi, Ross, McMeekin, & Roberts, 1996) and if possible easily interpretable parameters (Ross & Dalgaard, 2004).

In this chapter, we will first focus on the secondary modeling of the specific growth rate, developing some of the various approaches proposed. Then we will introduce the modeling of the lag time, the modeling of inactivation parameters, and the modeling of the probability of growth.

Secondary Models for Growth Rate

Models Based on the Gamma Concept

Many models are based on the square root model proposed by Ratkowsky et al. (1982) to describe the effect of the temperature T on the maximum specific growth rate $\mu_{\max}$:

$$\sqrt{\mu_{\max}} = b(T - T_{\min}) \tag{21.18}$$

where b is a constant and $T_{\min}$ the theoretical minimum growth temperature. Different models were proposed to extend the applicability of this model to temperatures near and above the optimal growth temperature (Ratkowsky, Lowry, McMeekin, Stokes, & Chandler, 1983; Zwietering, de Koos, Hasenack, de Wit, & van't Riet, 1991; Rosso, Lobry, & Flandrois, 1993). Among them the model developed by Rosso et al., named the cardinal temperature model, is one of the more often used models. It offers a great advantage to be characterized by parameters which have an obvious biological or graphical interpretability. These parameters are the minimum growth temperature $T_{\min}$, the maximum growth temperature $T_{\max}$, the optimal growth temperature T_{opt}, and the optimal specific growth rate μ_{opt} reached at this temperature. This commonly used model belongs to the family of the cardinal parameter models (Rosso and Robinson, 2001) and can be defined by Eqs. 21.19 and 21.20 as follows:

$$\mu_{\max}(T) = \mu_{\text{opt}} CM_2(T) \tag{21.19}$$

with

$$CM_n = \begin{cases} 0, & X \le X_{\min} \\ \dfrac{(X - X_{\max})(X - X_{\min})^n}{\left(X_{\text{opt}} - X_{\min}\right)^{n-1}\left[\left(X_{\text{opt}} - X_{\min}\right)\left(X - X_{\text{opt}}\right) - \left(X_{\text{opt}} - X_{\max}\right)\left((n-1)X_{\text{opt}} + X_{\min} - nX\right)\right]}, & X_{\min} < X < X_{\max} \\ 0, & X \ge X_{\max} \end{cases} \tag{21.20}$$

In this family the cardinal pH model (Rosso, Lobry, Bajard, & Flandrois, 1995) defined by Eq. 21.21 and the cardinal a_w model defined by Eq. 21.22 were also developed:

$$\mu_{\max}(\text{pH}) = \mu_{\text{opt}} CM_1(\text{pH}) \tag{21.21}$$

$$\mu_{\max}(a_w) = \mu_{\text{opt}} CM_2(a_w) \text{ with } a_{w\max} = 1 \tag{21.22}$$

Each of these models is often simplified as data points near the maximum parameter value are rare; in the cardinal pH model, the maximum growth pH (pH_{max}) is often fixed to $2pH_{opt} - pH_{min}$ and in the cardinal a_w model the parameter $a_{w\text{opt}}$ is often fixed to 1.

For the simultaneous modeling of the maximum specific growth rate μ_{max} as a function of more than one environmental factor, the gamma concept first introduced by McMeekin et al. (1987) and named by Zwietering, Wijtzes, de WIT, and van't Riet (1992) is often used. The gamma concept relies on the observation that in many conditions, environmental factors act independently on μ_{max} and that the global effect may be described by a multiplicative model. For example, a model describing the simultaneous effect of T, pH, and a_w may be simply written from previous equations as

$$\mu_{max}(T) = \mu_{opt} \times CM_2(T) \times CM_1(\text{pH}) \times CM_2(a_w) \quad \text{with } a_{w\,max} = 1 \qquad (21.23)$$

The gamma concept has been extended to take into account interactive effects between factors in conditions where such interactive effects are observed (Augustin & Carlier, 2000; Le Marc et al., 2002), but simple models based on the gamma concept are often sufficient to correctly describe the simultaneous effect of environmental factors (Lambert & Bidlas, 2007).

Models for specific growth rates are generally fitted by nonlinear regression to data points transformed in square root (on $\sqrt{\mu_{max}}$), using an additive Gaussian error model (Ratkowsky, 2004). In the R package nlstools, six models are proposed: the cardinal temperature model, the cardinal pH model in its two forms (three or four parameters), the cardinal a_w model in its two forms, and the complete model proposed by Pinon et al. (2004) with nine parameters (μ_{opt}, T_{min}, T_{opt}, T_{max}, pH_{min}, pH_{opt}, pH_{max}, $a_{w\,min}$ and $a_{w\text{opt}}$). As an example of fitting of such a model, Fig. 21.4 represents the fitting of the cardinal temperature model to a data set published by Tamplin, Paoli, Marmer, and Phillips (2005) concerning the effect of temperature on the growth of *E. coli* O157:H7 in raw sterile ground beef, using nlstools functions.

Polynomial Models

In the past, polynomial models were very often used to describe the simultaneous effect of various environmental factors. They were extensively used during the 1990 s and remain widely applied although models based on the gamma concept are now becoming popular. This extensive use is certainly due to the fact that polynomial models are very easy to fit by multiple linear regression, available in most statistical packages. The mathematical form of polynomial models is always the same and theoretically enables the fit of any data sets corresponding to various values of growth or inhibition parameters observed for different values of environmental factors. For example, the natural logarithm of the generation time, $\ln(t_g)$, was often modeled as a quadratic

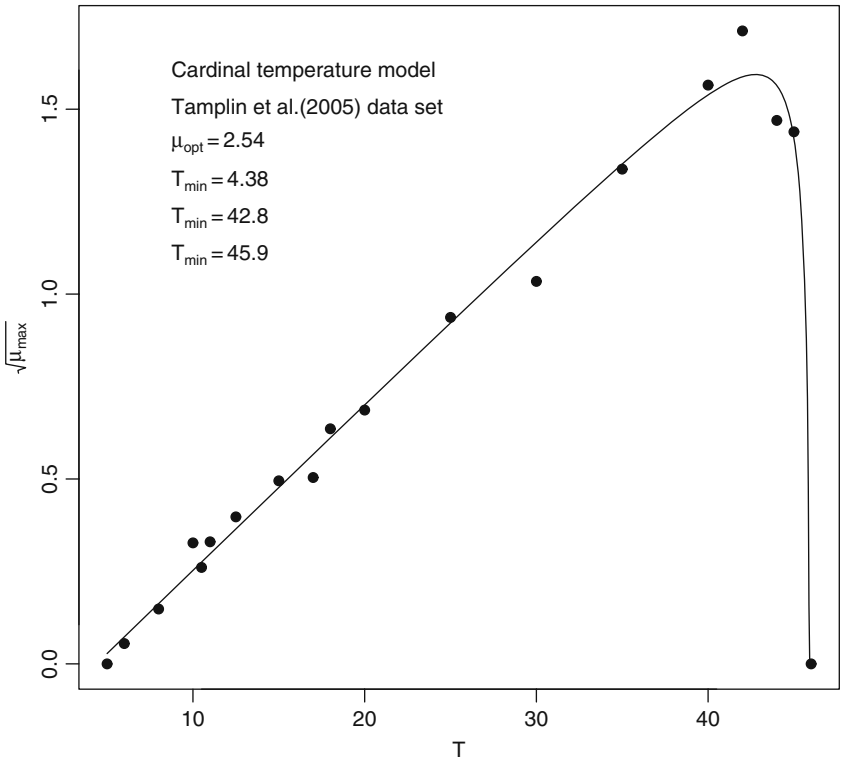

Fig. 21.4 Fitting of the cardinal temperature model to a data set published by Tamplin et al. (2005) concerning the effect of temperature on the growth of ***E. coli*** O157:H7 in raw sterile ground beef

polynomial function of environmental factors (for example, T the temperature, P the pH and S the NaCl percentage) by the following equation:

$$\ln(t_g) = b_0 + b_1 T + b_2 P + b_3 S + b_4 T^2 + b_5 P^2 + b_6 S^2 + b_7 TP + b_8 TS + b_9 PS \tag{21.24}$$

Such a model, also called a surface response model, is easy to fit to experimental data, whatever the number of environmental factors, and the fit can be easily improved when necessary by increasing the order of the polynomial equation. But the number of parameters to estimate from the data $(b_0, b_1, \ldots, b_k)$ rapidly increases with the number of environmental factors and with the order of the polynomial equation. In the past, cubic models with 35 parameters were sometimes proposed to predict the generation time as a function of 4 environmental factors (Buchanan & Bagi, 1994). Such flexible models have been criticized, as they attempt to model the experimental error

rather than eliminate it, and thus lack robustness (Baranyi et al., 1996). Their use should be restricted to a fit on huge data sets of very high quality and a predictive use only on the interpolation region of the experimental plan, which is smaller than the intuitive rectangular parallelepiped defined with the ranges of environmental factors, and not so easy to characterize (Baranyi et al., 1996). An approach was proposed by Geeraerd et al. (2004) to avoid drawbacks due to a too great flexibility, by developing polynomial models respecting biologically predefined constraints. But the fit of models with such an approach is no more easy, and thus polynomial models seem to lose their main quality.

Another drawback of polynomial models is that their parameters are coefficients $(b_0, b_1, \ldots, b_k)$ which are not easily interpretable. They have no direct biological meaning in contrary to parameters such as the minimum growth temperature $T_{\min}$. The result of the fit of a polynomial model on a data set may thus be really difficult to interpret. For example, the statistical significance of interaction coefficients (b_7, b_8, or b_9 in Eq. 21.24) is often interpreted as the proof of a biological interaction between corresponding factors, as it may be caused only by a bad global fit of the model to the data set (Lambert & Bidlas, 2007). As another consequence of the lack of interpretability of model parameters, different polynomial models are very difficult to compare.

In conclusion, due to the drawbacks previously described, the use of polynomial models should be restricted to cases where no other approach is possible, and the development and use of such models should be very cautious (Ratkowsky, 2004; Ross & Dalgaard, 2004).

Other Models

The previous presentation of existing models is not exhaustive. Some of the more commonly used models were presented, but many other models were proposed. Other models derived from the square root models were developed based on the gamma concept as the cardinal parameter models. Models derived from the Arrhenius equation were also proposed, but far less used than other models. The use of neural networks was also proposed to develop secondary models, but in this "black box" approach, the same drawbacks than those of polynomial models are found again. A complete review of secondary models may be found in the book "*Modelling Microbial Responses in Food*" edited by McKellar and Lu, in the corresponding chapter (Ross & Dalgaard, 2004).

Other Secondary Models

Secondary Models for Lag Time

The lag time λ corresponds to the time needed by bacterial cells to adapt to a new environment, for example after food contamination, before starting an exponential growth. This parameter is much more difficult to predict than the

maximum specific growth rate μ_{max}, as it does depend not only on current conditions but also on previous environmental conditions of the bacterial cells and on their physiological state. Many authors have reported a strong influence of the pre-incubation temperature on the lag phase duration (Swinnen, Bernaerts, Dens, Geeraerd, & Van Impe, 2004). As an example, bacterial cells previously cultured at low temperatures have a reduced lag at low temperatures compared with cells previously cultured at high temperatures (Membré, Ross, & McMeekin, 1999; Whiting & Bagi, 2002).

During the 1990 s, many authors modeled the lag time independently of the maximum specific growth rate (or generation time) (for a review, see Delignette-Muller, 1998). They generally proposed polynomial λ models developed from growth kinetics of cells previously cultured at a favorable high temperature. Consequently, when these models are used to predict the growth of an environment contaminant in a refrigerated food product, λ is overestimated. Membré et al. (1999) suggested that in such studies, microorganisms should be previously cultured at low temperatures, in order to mimic the processes of contamination in industry.

Other authors assumed that the product $h_0 = \mu_{max}\lambda$ does not depend on the growth conditions, but only on the pre-incubation conditions. Under this assumption, λ may be simply predicted from the predicted value of μ_{max} and from the constant h_0 for given pre-incubation conditions (Augustin & Carlier, 2000; Pinon et al., 2004). This product h_0 was described as the "work to be done" by the cells during the lag phase to prepare for the exponential growth (Robinson, Ocio, Kaloti, & Mackey, 1998; Pin, Garcia de Fernando, Ordonez, & Baranyi, 2002). But this product may be considered constant only in a first approximation (Delignette-Muller, 1998; Pin et al., 2002), and authors showed an increase of its value with the magnitude of the shift between two environmental conditions (Mellefont & Ross, 2003; Delignette-Muller et al., 2005; Mellefont, McMeekin, & Ross, 2005).

Another difficulty encountered while trying to develop predictive models for the lag time is that the observed population lag time depends on the inoculum level. The population lag time decreases with the initial number of cells for law inocula, such as those that may be encountered in realistic food contaminations. As this observed effect is due to the variability among individual cell lag times (Baranyi, 1998; Augustin, Brouillaud-delattre, Rosso, & Carlier, 2000), a predictive model should take account of this variability by describing the distribution of individual lag times. Models were recently proposed to describe the distribution of individual lag times and the effect of environmental factors on this distribution (Francois et al., 2005; Guillier & Augustin, 2006; Standaert et al., 2007), but these models still need to be compared and validated before an extensive use.

In conclusion, considering the modeling of environmental factors on the lag time, more research is still required before secondary models as predictive as the growth rate models can be proposed for lag time.

Secondary Models for Inactivation

The Bigelow model has been a standard for decades to predict the effect of temperature on the classical decimal reduction time D (D-value), and this model was also more recently used to model in the same way the time of the first decimal reduction, δ, estimated from the fit of the Weibull inactivation model (Mafart et al., 2002). The Bigelow model simply describes the logarithm of the ratio between the D-value at the temperature T and the D-value at a reference temperature T_{ref} as a linear function of the difference $T - T_{\text{ref}}$ by the equation

$$\log_{10}\left(\frac{D}{D_{\text{ref}}}\right) = -\frac{T - T_{\text{ref}}}{z} \tag{21.25}$$

The parameter z corresponds to the reciprocal of the slope and is commonly named the z-value. Such a model may be very easily fitted by simple linear regression on the logarithm of the D-values. This model has been used for decades to calculate processing times for thermal processes such as pasteurization and sterilization.

Only few models were developed to predict the effect of multiple environmental factors on inactivation parameters, and most of them are polynomial models (Ross & Dalgaard, 2004). Some attempts were made to extend the Bigelow model by describing the effect of other environmental factors on Weibull inactivation parameters using a progressive approach such as the one proposed in the gamma concept (Couvert, Gaillard, Savy, Mafart, & Leguérinel, 2005; Leguérinel et al., 2007). More research is needed in its field in order to propose predictive models taking into account factors other than temperature that may contribute to inactivation.

Probability Models

Several types of models were proposed to describe the growth/no growth limit as a function of various environmental factors. A deterministic modeling of this limit may be deduced from secondary growth models based on the gamma concept (Lambert & Bidlas, 2007) from the characterization of parameters such as the minimum growth temperature (T_{min}), taking into account if necessary interaction between environmental factors (Augustin & Carlier, 2000; Le Marc et al., 2002). A stochastic modeling of this limit may also be performed using logistic regression to model categorical data (growth or no growth) as a function of various environmental factors. This use of logistic regression in predictive microbiology was introduced by Ratkowsky and Ross (1995) and used by many authors.

In logistic regression, the logit function of the probability of growth ($\text{logit}(p) = \ln(p/(1-p))$) is described as a function of the environmental factors. Two types of models are commonly used: the first describing $\text{logit}(p)$ as a polynomial function such as the one of Eq. 21.24 and the second describing

logit(p) as a nonlinear function based on models developed using the gamma concept (Ross & Dalgaard, 2004; Gysemans et al., 2007). The second approach should be preferred when such models give reasonable fits to data, for the reasons already developed when dealing with μ_{max} models. It gives more robust models, which are parameterized with interpretable parameters (Gysemans et al., 2007). Nevertheless, the first approach with polynomial functions might be used for data sets showing nontypical growth limits, such as those observed by Gysemans et al. (2007) on mixed strain data.

As for modeling the lag time, the potential effect of the past of the microbial cells and of the inoculum should be considered in experimental works aimed at characterizing the growth/no growth limit. As for the lag time, the variability among individual cells may have a greater impact on this limit for low inocula (Skandamis et al., 2007). Indeed, at a given environmental condition, the larger the cell population, the more likely it is to contain at least one cell that is capable of initiating growth. Concerning the potential impact of the past of the cells, Skandamis et al. (2007) reported an impact of a preliminary acid adaptation of *E. coli* O157:H7 cells on their growth/no growth limit, but further researches are needed before models could take such impacts into account.

Applications of Predictive Modeling

The Challenge of Modeling Microbial Dynamics in Food Under Realistic Conditions

Models developed from microbial experiments in laboratory conditions must be validated in realistic conditions before being used as predictive models. Validation studies must demonstrate that microorganisms in food products behave in a similar way as in laboratory conditions. Users of such models must be aware of the limitations of their applicability.

The models previously described were often developed from data obtained from a culture of one or more strains of one species, in liquid media, and in constant environmental conditions. To be able to predict survival and growth of microorganisms in realistic conditions, these models must be extended to take into account the effect of changing environmental conditions, of the potential interaction between various strains from different species developing in a same food product and of the effect of the structure of nonliquid food products.

From a mathematical point of view, it is not difficult to predict microbial growth with changing environmental conditions, by numerically integrating the differential equations defining the growth model, as first proposed by Baranyi, Robinson, Kaloti, and Mackey (1995). This is generally done assuming that the primary model parameters (μ_{max}, for example) immediately change according

to the changing environmental factors and the secondary model. This hypothesis seems to be reasonable to predict microbial growth under nonisothermal conditions, as far as the thermal scenario is sufficiently smooth, but delayed responses may be observed for sudden strong fluctuations of the temperature (Baranyi et al., 1995; Swinnen, Bernaerts, Gysemans, & Van Impe, 2005). The use of this procedure has been validated several times for food products under realistic nonisothermal conditions, especially in meat products (Mataragas, Drosinos, Siana, Skandamis, & Metaxopoulos, 2006; Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006). The same type of procedure may be used to predict microbial inactivation, using the same biological hypothesis. Its use was not so extensively validated than for growth prediction. It was recently successfully validated for nonisothermal realistic conditions (Aragao, Corradini, Normand, & Peleg, 2007) and high-pressure-changing conditions (Koseki & Yamamoto, 2007), but it was shown by other authors that this procedure may overestimate the effect of an applied heat treatment as not taking into account the potential physiological adaptation of the microorganisms (Bernaerts et al., 2004; Valdramidis, Geeraerd, & Van Impe, 2007).

Concerning the potential effect of the natural flora of the food product and of its structure, some attempts were made to model them in some specific cases. The interaction between food flora and a pathogen microorganism may, in some cases, be modeled by a simple competition between both populations (Vimont et al., 2006; Mellefont, McMeekin, & Ross, 2008), but other more complex interactions may also be observed in food. A few models were proposed to describe growth interactions involving the diminution of the pH or the production of bacteriocins due to microbial growth (Janssen et al., 2006; Leroy & De Vuyst, 2007), but much work still needs to be done before proposing a global modeling approach of microbial interactions. Concerning the effect of food structure, a first model was recently proposed, but much work still needs to be done on this subject too (Antwi, Bernaerts, Van Impe, & Geeraerd, 2007). As very complex phenomena might occur during microbial growth in food products, one should keep in mind that a preliminary validation of the use of models in the food product of interest and in realistic environmental conditions is required to ensure reasonable predictions.

Quantitative Microbiology Tools

The use of predictive models is of great interest to improve the microbiological safety of food products, but among the large number of published models, only a minority of them is electronically accessible from simulation tools. For the other models, it is always possible to develop its own simulation tool from the publications, but this requires some more time and expertise from the user. Some simulation tools in predictive microbiology are freely provided. The

Pathogen Modeling Program (PMP) has been developed by the US Department of Agriculture–Agricultural Research Service (USDA–ARS) since the 1990 s. It includes models for growth and inactivation and is updated from time to time to include newly developed models. It may be freely downloaded from the USDA–ARS web site (http://ars.usda.gov/services/software/software.htm). The Growth Predictor is the successor to the Food MicroModel program and has been developed by the UK Institute for Food Research. It provides only growth models as indicated by its name. It may be freely downloaded from the UK Institute of Food Research web site (http://www.ifr.ac.uk/Safety/Growth Predictor). The Pathogen Modeling Program and the Growth Predictor include models that were developed in broth media and not necessarily validated in food products. A preliminary validation of simulations in the food product of interest and in realistic environmental conditions is thus essential for a safe use. For this validation, the user may use its own experimental data and/or published data. The search for published data corresponding to a specific microbial species, a specific food product, and a set of environmental factors is facilitated by the use of the ComBase database. ComBase is a very large, freely available repository of microbiological data for predictive microbiology, accessible from the web (http://wyndmoor.arserrc.gov/combase/). Some few programs providing simulations validated on food products exist. The SeafoodSpoilage and Safety Predictor (SSSP) has been developed by the Danish Institute for Fisheries Research and is freely accessible to simulate the spoilage of seafood products (http://www.difres.dk/micro/sssp/). The Sym'Previus program (http://www.symprevius.org/) is a simulation tool that includes microbiological data from literature, published models, data from challenge tests in food products, but its use is not free and is restricted to registered users.

Time–temperature integrators or indicators (TTI) may be seen as another type of tool from quantitative microbiology. Such tools have been developed from the 1990 s in order to record the temperature with time and translate its effect on the microbial growth in the product of interest in easily readable information such as a color indicator. A TTI is a simple inexpensive device that indicates the temperature history in terms of microbiological status of the food product. Such devices may be based on mechanical, chemical, enzymatic, or microbiological systems that produce a color change as much rapidly as the temperature is high (Taoukis, Koutsoumanis, & Nychas, 1999; Vaikousi, Biliaderis, & Koutsoumanis, 2008). Applications of TTI to optimize the microbiological control of fish or meat products were reported (Moore & Sheldon, 2003; Koutsoumanis et al., 2006).

Predictive Models for Quantitative Risk Assessment

Quantitative microbiological risk assessment (QMRA) has become a classic approach in food microbiology, generally divided into four stages: hazard

identification, hazard characterization, exposure assessment, and risk characterization. The use of models from predictive microbiology is often essential in the exposure assessment. Predictive models used in an exposure assessment should have been previously validated and the different sources of variability and uncertainty in these models should be clearly stated (Nauta, 2007). Indeed both uncertainty and variability may lead to imprecise model predictions, and each one should be characterized separately. Variability refers to true heterogeneity of the population considered and cannot be reduced by additional data, while uncertainty refers to lack of knowledge and may be reduced by the acquisition of additional data. Variability may correspond to variability between strains of the same species, that may have a great impact on the results of a QMRA (Delignette-Muller & Rosso, 2000), or variability in the composition of the food and in the growth conditions not included in the model as environmental factors (Delignette-Muller, Cornu, Pouillot, & Denis, 2006). Uncertainty may rely on the choice of the good model and on the estimation of the parameters of the chosen model, especially due to a lack of data. One should be aware that variability on predictive models' parameters is often underestimated, as microbiological practice tends to reduce sources of variability by standardizing experiments in order to reach reproducible results. New approaches, such as the use of Bayesian inference, may help to quantify the variability and uncertainty on parameters of predictive models from data from disparate sources (Pouillot, Albert, Cornu, & Denis, 2003; Delignette-Muller et al., 2006).

Conclusion

Much work has been done in predictive microbiology during the last 30 years but much work still needs to be done. Even if a great number of models have been published, a minority of them has been included in freely provided simulation tools, and only a small number of them have been clearly validated for predictions in food products under realistic conditions. Nowadays, a safe use of these models thus often requires a good background in modeling and the acquisition of some data on the food products of interest. An effort has been done by the researchers in predictive microbiology to facilitate the use of models and the access to microbiological data, but this effort should be maintained in order to make the use of models even more easy and safe. Moreover, some research fields in predictive microbiology, such as modeling of microbial interactions or modeling of the effects of food structure, have just emerged and should give interesting results in the next years. New trends may also emerge in predictive microbiology from links with other research fields such as genomics, bioinformatics, or systems biology.

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Chapter 22
Predictive Modeling of Pathogen Growth in Cooked Meats

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Introduction

Thermal processing or cooking of food products has been adopted for centuries as a method of food preservation. Enhancement of product quality parameters such as color, flavor, and texture probably contributed to the adoption of the method for a variety of products. Today, cooking or thermal processing is one of the most commonly used unit operation in the food industry. The significant advantages to cooking of meat and poultry products include extension of shelf life, desirable organoleptic properties, enhanced economic value, and assurance of safety of the products.

A variety of cooking technologies has been used in the meat industry, including traditional thermal processing (moist or dry heat, or a combination), microwaves, radio frequencies, infrared, and combination treatments. The cooking methods adopted for a specific product depend on the raw materials to be used, ingredients used, and the end product characteristics desired. Table 22.1 provides a list of various categories of processed, ready-to-eat (RTE) meat and poultry products, including cooked meats. In most traditional cooked meat and poultry products, the meats are subjected to moist heat to raise the temperature of the product, and subsequently, dry heat is applied to remove moisture from product surface. In some cases, the sequence of the heating protocols is reversed to form a cooked surface on the product and subsequently heat is delivered using moist heat with different levels of humidity.

Cooked meat and poultry products can be broadly categorized into either pasteurized or sterilized products, depending on the degree of heat applied to the product. Most of the cooked products are pasteurized, with the applied heat sufficient to destroy vegetative spoilage organisms and foodborne

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Table 22.1 Classification of some processed, ready-to-eat (RTE) meat and poultry products

Dried products:
Basturma, pastirma, basturmi, beef sticks, carne seca, dried beef, dry duck breast, meat/poultry jerky
Salt-cured products:[1]
Cappicola, coppa, country ham, dry cured duck, Parma ham, prosciutto, prosciutti
Fermented products:[2]
Alessandri (dry sausage), apenino (dry sausage), Arles or D'Arles (dry sausage), bockwurst (semi-dry sausage), cacciatore/cacciatora (dry sausage), cervelat, soft cervelat, chorizo, Lebanon bologna, pepperoni, soft salami, salami: Genoa, Italian, German, summer sausage, thuringer, soft thuringer
Cooked or otherwise processed whole or comminuted products:
Meat: Berliner (cooked, smoked sausage), bologna, cooked bratwurst, Braunschweiger/liver sausage, breakfast link sausage or patties, brown and serve sausage, burritos, cheese smokies, cheesefurter, cheesewurst/cheddarwurst, chili, chorizo, cooked beef, cooked ham, cooked pork in BBQ sauce, cotto salami, Fleischkaese (cured, cooked sausage), frankfurters, gyros, meat loaf, meat salads, frozen meat soups, nem chua (cooked, pickled ham with shredded pork skin), pasta with meat sauce, pastrami, pickled pigs feet in vinegar, pickled sausages/meat in vinegar, piroshki, pork barbecue, pork sausage patties, ravioli, roast beef, roast pork, souse, stews, white hots, Weiners
Poultry (includes products containing any amount of poultry): chicken burritos, chicken BBQ, chicken bologna, chicken breast, chicken franks, cooked poultry, cooked poultry rolls, corn chowder with chicken, poultry loaf, poultry patties, poultry rolls, frozen poultry soups, turkey BBQ, turkey franks
Thermally processed, commercially sterile products:
Canned spaghetti with meatballs, canned corned beef hash, canned ham, canned chicken salad, canned soups with meat or poultry

[1]Majority of these products do not undergo any thermal process.
[2]Most of these products originated in Europe and as processed in Europe, they do not receive any heat treatment. However, the US versions of many of these products receive a mild heat treatment.

pathogens. Spores of the spore-forming organisms can survive this process, and depending on the cooling rate and the product characteristics (a_w, pH, ingredients, etc.), these organisms can germinate and grow. Thus, the cooling of such products should be designed to prevent the germination of these organisms or should be formulated (with antimicrobials) to prevent or inhibit the heat-shocked (activated) spores from germination and outgrowth. In addition, the product characteristics should prevent the surviving spores from growth during subsequent storage conditions employed during distribution and by the consumer at home.

While the performance standards for lethality of meat and poultry products are specified by product type by the regulatory agencies, the lethalities achieved traditionally by the ready-to-eat (RTE) meat and poultry processors exceed them. For example, the performance standard for lethality for *Salmonella* spp. destruction during processing of RTE poultry is 7.0 $\log_{10}$, while that for other RTE products is 6.5 $\log_{10}$ reduction (USDA-FSIS, 1999). Typical

cooking processes employed for thermally processed meats achieve internal temperatures of ≥68°C (154°F), which would destroy majority of the non-spore-forming pathogens, *Salmonella* spp., *Escherichia coli* O157:H7, *Staphylococcus aureus*, and others, including *Listeria monocytogenes* (Ranken, 2000). Thus, majority of the flora in processed products would represent microorganisms resulting from recontamination of the products. In addition, the performance standards for stabilization require no growth of *Clostridium botulinum* and no more than 1 $\log_{10}$ growth of *Clostridium perfringens* throughout all RTE meat and poultry products (USDA-FSIS, 1999).

Sterilization refers to complete destruction of microorganisms, including the spores of the most resistant spore-forming organisms, regardless of the pathogenicity of the organisms. However, in the food processing context, commercial sterility is achieved, wherein the organisms capable of growing in the product under non-refrigerated conditions (>10°C) are destroyed. Traditionally, this refers to a process similar to sterilization, where all the spores of pathogenic species are destroyed, while spores of resistant, thermophilic spoilage organisms may survive, but do not grow under normal storage and distribution conditions. The terms shelf stability and commercial sterility are interchangeably used in the USA in meat and poultry processing. Typical processes include an F_o value of 3 (3 min at 121°C), although typical commercial processes exceed this requirement and achieve an F_o of 5–8.

While the pasteurization and sterilization can be considered two extremes of thermal processing, a third category, *sous vide* (under vacuum) processing, has gained popularity in Europe and several products are currently in the market within this category. These products are characterized by the application of a combination of mild thermal processing and vacuum packaging to preserve meat products, while assuring safety and preserving the sensory characteristics of the product (Hyytiä-Trees et al., 2000). The thermal processes applied normally include heating the products to 70°C for 100 min or 90°C for 10 min followed by storage and handling at refrigeration temperatures (Grant & Patterson, 1995). The microbiological safety risks associated with *sous vide* meat and poultry products are the survival and potential growth of psychrotrophic foodborne pathogens and spore-forming organisms including *C. botulinum* that could survive the mild heat treatment and potential germination and outgrowth during storage (Hyytiä-Trees et al., 2000). The safety of a variety of *sous vide* products has been studied with respect to *C. botulinum* and *Bacillus* spp. (Fain et al., 1991; Hyytiä-Trees et al., 2000; Betts, 1998; Church, 1998; Ghazala & Trenholm, 1998; Juneja, 1998; Nissen, Rosnes, Brendehaug, & Kleiberg, 2002). The recommended thermal processes, related product shelf life, and the target organism for processing of *sous vide* products are provided in Table 22.2. A survey of commercially available *sous vide* products concluded that the health risks associated with these products are quite low as long as very low storage temperatures are maintained (Nissen et al., 2002).

Table 22.2 Recommended heat treatments for *sous vide* products[1]

Source	Pasteurization treatment (internal temperature)	Shelf life (under refrigeration)	Target organism
French regulations (Ministère de l'Agriculture 1974, 1988)	70°C for 40 min	6 days	*Enterococcus faecalis*
	70°C for 100 min	21 days	
	70°C for 1000 min	42 days	
DOH guidelines (1989)	70°C for 2 min	5 days	*Listeria monocytogenes*
SVAC (1991)	80°C for 26 min or 90°C for 4.5 min	8 days	*Clostridium botulinum* type E
ACMSF (1992)	90°C for 10 min	>10 days	*C. botulinum*
ECFF Botulinum Working Party (Gould, 1996)	70°C for 2 min	Short shelf life	*L. monocytogenes*
	90°C for 10 min	Longer shelf life	*C. botulinum*

[1]Adapted from Creed (1998).

Foodborne Pathogens of Significance in Ready-to-Eat Meat and Poultry Products

The microorganisms of concern with respect to RTE meat and poultry products include spores of spore-forming pathogens *C. botulinum*, *C. perfringens* and *B. cereus* during cooling and holding of products and *L. monocytogenes* resulting from recontamination of thermally processed products. Within the *sous vide* meat and poultry products, the survival and growth of non-proteolytic *C. botulinum* is of significance as these spores can germinate and grow during extended refrigerated storage and distribution. Consequently, the organisms of significance in cooked, RTE meat and poultry products, their characteristics, and importance will be discussed briefly.

Clostridium botulinum

C. botulinum comprise a group of Gram-positive, spore-forming, rod-shaped, anaerobic bacteria that produce a potent neurotoxin. The *C. botulinum* species are differentiated based upon the antigenically specific toxins that they produce. Thus, there are currently seven known types A–G, with type C having C1 and C2 toxins. All the strains of *C. botulinum* are at present placed in four groups. Group I contains proteolytic types A, B, and F; Group II, the non-proteolytic types B, E, and F; Group III, the types C and D; and Group IV, the type G. Group I contains the proteolytic type A, B, and F strains (Cato, George, & Finegold, 1986; Gibson & Eyles, 1989; Hatheway, 1993). Botulism in humans is

predominantly caused by Group I and Group II strains. The *C. botulinum* Group I are actively proteolytic, digesting the native proteins of meat, milk, and egg white and liquefy gelatin, while Group II are non-proteolytic, but capable of liquefying gelatin, however less actively than Group I. The optimal growth temperature for Group I strains is 35– 40°C and the minimum temperature is 10°C (Lund & Peck, 2000; Parkinson & Ito, 2006).

Foodborne botulism is an intoxication and results from the ingestion of preformed toxin produced in food by *C. botulinum*. Majority of the human botulism cases worldwide are caused by ingestion of food containing preformed toxins of types A, B, and E. Fewer than 10 incidents of foodborne type F botulism in humans have been documented, and there are reports of two incidents attributed to organisms producing type C toxin and one to an organism producing type D toxin (Smith & Sugiyama, 1988). The US Centers for Disease Control and Prevention (CDC) documented 724 cases of verified foodborne botulism in US adults from 1973 to 1996 (Shapiro, Hatheway, & Swerdlo, 1998).

The *C. botulinum* spores are widespread in the soil, with soils of wet sediments and shorelines containing more numbers of spores than inland soils (Dodds, 1993). *C. botulinum* is ubiquitous and may occur in almost all foods, whether of vegetable or animal origin. The *C. botulinum* types most commonly associated with meats are A or B. Foods commonly implicated in botulism are fish, meat, and vegetable products. Other types of foods such as cheese, hazelnut yoghurt, peanuts, and black olives have also been involved in the food poisoning outbreaks. Prevalence of *C. botulinum* spores in meats in North America is low, with an average MPN of 0.1 spore/kg, while in Europe, it is higher (2.5 spores/kg) (Austin & Dodds, 2001).

Consumer demand for prepared foods of high quality, containing minimal or no preservatives, and are minimally processed has led to the development of *sous vide* and cook-chill foods. In Europe, these foods are also called refrigerated processed foods of extended durability (REPFEDs). The normal thermal process these foods are subjected to is considerably lower (70–95°C) than the canned foods, to preserve organoleptic properties of the foods. Subsequent to the thermal process, the food is cooled rapidly and stored at refrigeration temperatures (1–8°C). These foods are not sterile, and product shelf life is dependent on the heat treatment applied, storage temperature, and the intrinsic properties of the food (e.g., pH, water activity). Recommended processing practices for minimally heated, chilled foods (REPFEDs) to assure their microbiological safety are provided in Table 22.3. While the typical shelf life of such foods can be up to 42 days, in the USA, a more severe thermal process (95–100°C for 60–90 min) is applied, with >90 day shelf life.

Concerns associated with *sous vide* and cook-chill foods involve the microbiological safety of the products (Rhodehamel, 1992), particularly the psychrotrophic, spore-forming foodborne pathogens (non-proteolytic Group II *C. botulinum*). Mild heat treatments in combination with vacuum packaging may actually select for *C. botulinum* and increase the potential for botulism. Growth

Table 22.3 Processing practices to ensure the safety of minimally heated, chilled foods with respect to non-proteolytic *Clostridium botulinum* (modified from Peck, 2006)

1. Storage at <3°C[1]
2. Storage at ≤5°C and a shelf life of ≤10 days
3. Storage at 5–10°C and a shelf life of ≤5 days
4. Storage at chill temperature[2] combined with heat treatment of 90°C for 10 min or equivalent lethality (e.g., 70°C for 1675 min, 75°C for 464 min, 80°C for 129 min, and 85°C for 36 min)[3]
5. Storage at chill temperature combined with ≤pH 5.0 throughout the food
6. Storage at chill temperature combined with a salt concentration ≥3.5% throughout the food
7. Storage at chill temperature combined with $a_w \leq 0.97$ throughout the food
8. Storage at chill temperature with combinations of heat treatment and other preservative factors, which can be shown consistently to prevent the growth and toxin production by *C. botulinum*

[1]Originally 3.3°C but growth has now been demonstrated at 3.0°C (Graham et al. 1997).
[2]Chill temperature is specified as 8°C in the United Kingdom.
[3]Alternative heat treatments of 80°C for 270 min and 85°C for 52 min are recommended by the European Chilled Food Federation (ECFF, 1996)

and survival properties of proteolytic and non-proteolytic *C. botulinum* are provided in Table 22.4.

However, the temperature control in chill chains is often inadequate, and temperature abuse is common throughout distribution and retail markets and by consumers (Daniels, 1991; Harris, 1989; Kalish, 1991). The Advisory

Table 22.4 Growth and/or survival properties of proteolytic and non-proteolytic *Clostridium botulinum* (Peck, 2006)

	Proteolytic *C. botulinum*	Non-proteolytic *C. botulinum*
Neurotoxins formed	A, B, F	B, E, F
Minimum temperature for growth	10–12°C	2.5–3.0°C
Optimum temperature for growth	37°C	25°C
Minimum pH for growth	4.6	5.0
NaCl concentration preventing growth	10%	5%
Minimum water activity for growth		
NaCl as humectant	0.96	0.97
Glycerol as humectant	0.93	0.94
Spore heat resistance	$D_{121°C} = 0.21$ min	$D_{82.2°C} = 2.4$ or 231 min[1]
Foods involved in botulism outbreaks	Home-canned foods, faulty commercial processing	Fermented marine products, dried fish, vacuum packed fish
Potential food problems	Canned foods	Minimally heated, chilled foods

[1]Heat resistance data without or with lysozyme during recovery.

Committee on the Microbiological Safety of Food (ACMSF, 1992) recommended certain procedures to ensure the safety of refrigerated processed foods of extended durability. According to these recommendations, heat treatments or combination processes should reduce the number of non-proteolytic *C. botulinum* bacteria by a factor of 10^6 (a 6-decimal [6-D] process). However, the capability of a combination process to consistently prevent growth and toxin production by *C. botulinum* in a particular product must be reliably demonstrated.

Clostridium perfringens

C. perfringens is a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium that is encapsulated and non-motile. Although an anaerobe, the organism can tolerate some exposure to air (Labbe, 2000). Five types of *C. perfringens* (A, B, C, D, and E) have been classified based on the production of enterotoxins and four other types of extracellular toxins, α (alpha), β (beta), ϵ (epsilon), and ι (iota). Type A strains are predominantly involved in foodborne toxico-infections of human beings. The primary toxin produced by *C. perfringens* is called type A enterotoxin (*C. perfringens* enterotoxin, CPE) and is responsible for the acute diarrhea, the predominant symptom of *C. perfringens* food poisoning (Bhunia, 2008). The toxin is produced in significant amounts in the intestines only and is associated with sporulation (Granum, 1990).

Meat and poultry products are generally implicated in most outbreaks, with beef products responsible for about 40% of *C. perfringens* foodborne outbreaks (Bhunia, 2008). Roast beef is a major vehicle of outbreaks because of improper handling, temperature abuse, and inadequate cooling after cooking. The CDC estimated that 248,520 foodborne illnesses due to *C. perfringens* occur in the USA annually (Mead et al., 1999).

C. perfringens is widely distributed in soil, dust, vegetation and in raw, dehydrated, and cooked foods. It is part of the normal flora of the intestinal tract of man and animals and is found in a large variety of foods of animal origin such as poultry, fish, dairy products as well as other foods, such as soups and gravies, spices, milk, and gelatin (ICMSF, 1996b; Labbe & Juneja, 2006). *C. perfringens* prevalence of 29, 60, and 35% on beef, pork, and lamb carcasses, respectively, was reported, although at low levels ($<$20 CFU/100 cm^2; Smart, Roberts, Stringer, & Shah, 1979).

C. perfringens is capable of growth between 15 and 50°C, with an optimum growth temperature between 43 and 46°C, while growth at $\leq$15°C is slow. *C. perfringens* is sensitive to low-temperature storage, with reduction in populations reported in meat products during refrigerated storage. *C. perfringens* grows best at pH values between 6.0 and 7.0 with a range of pH between 5.0 and 9.0. The lowest water activity supporting the growth of *C. perfringens* is 0.97, 0.95, 0.93, and 0.96 when sucrose, NaCl, glycerol, and glucose are used as

humectants, respectively. A level of 7–8% NaCl is required to prevent the growth of most strains although some inhibition occurs at levels of 5–6% NaCl (ICMSF, 1996b; Labbe, 2000; Labbe & Juneja, 2006).

The ability of *C. perfringens* spores to survive traditional thermal processing procedures and unusually short generation times (<10 min in meat) at temperatures between 43 and 46°C contributes to its role in outbreaks of foodborne illness caused by this organism. The heat resistance of *C. perfringens* spores varies widely. The $D_{95°C}$ values of heat-resistant and heat-sensitive spores of *C. perfringens* were reported to be between 17.6–63 min and 1.3–2.8 min, respectively (Ando, Suzuki, Sunagawa, & Oka, 1985). The heat treatments commonly used in meat processing provide the heat shock required by the spores to germinate and multiply, resulting in rapid growth if the products are not cooled to temperatures below 24.6°C, and subsequently to below 10°C.

The increasing demand and availability of minimally processed and *sous vide* foods present opportunities for the growth of *C. perfringens* in products that have not been associated with it previously (Labbe, 2000).

Bacillus cereus

B. cereus is a Gram-positive, rod-shaped, spore-forming bacterium that can grow both aerobically and anaerobically. *B. cereus* causes two distinct types of foodborne syndromes, emetic and diarrheal, which differ in the nature of disease as well as causative agent. The emetic syndrome is characterized by short incubation periods (1–5 h) and is caused by a small cyclic peptide primarily associated with starch-based foods and those containing starch, although other foods such as beef, poultry milk, and infant formula have also been reported (Granum & Baird-Parker, 2000; Agata, Ohta, Mori, & Isobe, 1995). On the other hand, the diarrheal syndrome is characterized by slightly longer incubation periods of 8–16 h. It is caused by the production of enterotoxins during vegetative growth of *B. cereus* in the small intestines following ingestion of vegetative cells and spores (Granum, 1994; Granum, Bryenstad, & Kramer, 1993). The diarrheal syndrome is mostly associated with meats, fish, vegetables, soups, sauces, and dairy products.

The spores of *B. cereus* occur widely in soils and may be found in virtually all raw and processed foods (Granum & Baird-Parker, 2000). It has been isolated from a wide variety of foods especially of plant origin, but also from meat, fish, and dairy products (Kramer, & Gilbert, 1989). Low numbers of this bacterial species can be found in a number of food products, including fresh and processed foods. *B. cereus* isolation rates of 6.6, 18.3, and 39.1% were reported in raw meats, meat products, and food ingredients, respectively, with levels of 2–4 log CFU/g (Konuma, Shinagawa, & Tokumaru, 1988).

Most *B. cereus* strains are mesophilic and can grow between 15 and 50 or 55°C. The optimal growth temperature ranges from 3 to 40°C (ICMSF, 1996a).

Psychrotrophic strains of *B. cereus*, able to grow at 4–5°C but not at 30–35°C, have been reported. The psychrotrophic nature of the organism is of concern in pasteurized milk and prepared chill-stored meals such as *sous vide*. *B. cereus* can grow over a wide pH range of 5.0–8.8 with pH optima between 6.0 and 7.0. In the presence of NaCl as a humectant, *B.* cereus will not grow at a_w of 0.93. However, when glycerol was used as a humectant, growth was observed at 0.93 a_w, but not at 0.92. The spores of *B. cereus* are moderately heat resistant ($D_{121°C}$ of 0.03 min), but some are markedly heat resistant ($D_{121.1°C}$ of 2.35 min). The ability to form spores ensures survival through all stages of processing short to retorting (ICMSF, 1996a).

The combination of abundance and ubiquity, heat resistance, and psychrotrophic nature makes (*B. cereus*) difficult to control in food processing environment. Effective destruction of spores is the ultimate goal for safe foods with an extended shelf life.

Listeria monocytogenes

L. monocytogenes is a small (0.5 μ in diameter and 1–2 μ in length), motile, non-spore-forming, Gram-positive, rod-shaped bacterium (Rocourt & Buchrieser, 2007). It is a facultative anaerobe and can grow in vacuum-packaged products, at refrigerated temperatures (psychrotrophic).

L. monocytogenes has been recognized as a human pathogen since 1929 and it is now recognized that nearly all cases of human listeriosis are foodborne (Adak, Long, & O'Brien, 2002; Mead et al., 1999). The risk of listeriosis is greatest among well-defined high-risk groups, including pregnant woman, neonates, and immune-compromised adults but may occasionally occur in persons who have no predisposing underlying condition (Painter & Slutsker, 2007), with a mortality of approximately 20% (Gellin & Broome, 1989). The perinatal infections in humans are manifested as abortions, still birth, neonatal sepsis, and meningitis. In adults, meningitis and encephalitis are the most common clinical symptoms, although other symptoms have been attributed to listeriosis. Most large human listeriosis outbreaks have been associated with *L. monocytogenes* serotype 4b strains, with other serotypes (1/2a, 1/2b, and 3a) (Farber, Daley, MackKie, & Limerick, 2000; Frye et al., 2002) reported to be associated with listeriosis in a few outbreaks (Gilbert, Mclauchlin, & Velani, 1993; Jacquet et al., 1995).

L. monocytogenes is widely distributed in nature and has been isolated from soil, water, vegetation, sewage, animal feeds, farm environment, and food processing environments (ICMSF, 1996c; Sauders & Wiedmann, 2007). *L. monocytogenes* has been isolated from a variety of foods including dairy, vegetables, poultry, sea foods, and meat and meat products. The contamination of RTE meat and poultry products by *L. monocytogenes* can originate from the processing environment of the chilling or slicing rooms (Pociecha, Smith, &

Manderson, 1991; Van der Elen & Snijders, 1993), personnel (Kerr, Birkenhead, & Seale, 1993), and processing equipment (food contact) (Lawrence & Gilmour, 1995). An exhaustive review of the prevalence of *Listeria* species in various meat products is presented by Farber, Pagotto, & Sherf (2007). *L. monocytogenes* can tolerate high salt (up to 20%) (Larson, Johnson, & Nelson, 1999) concentrations, can multiply over a wide range of temperatures (1–45°C) (Hudson, Mott, & Penney, 1994; Petran & Zottola, 1989), and tolerate a wide pH range (4.0–9.6) (Petran & Zottola, 1989; Phan-Thanh, 1998) and a_w (>0.90) (Johnson, Doyle, Cassens, & Schoeni, 1988; Larson et al., 1999; Miller, 1992; Nolan, Chamblin, & Troller, 1992). Thus, the traditional hurdles employed by the RTE meat industry of refrigerated storage, vacuum packaging, and inclusion of salt and nitrite in product formulations have minimal effect on controlling *L. monocytogenes* growth in such products. The USDA-FSIS and US Food and Drug Administration (FDA) risk assessment identified RTE meat and poultry products and seafood, especially the ones that are not reheated before consumption, as high-risk products for *L. monocytogenes* contamination and foodborne illness. Among the RTE meat and poultry products, hot dogs (frankfurters) and deli meats were identified as highest risk products for foodborne illness from *L. monocytogenes*.

The ability of *L. monocytogenes* to colonize, grow, and persist in the food processing environment and on food processing equipment reflects its ability to survive in the natural environment for extended periods.

Predictive Models to Describe Pathogen Growth in Cooked Meats

Predictive microbiology offers various tools in the form of mathematical models that can be handy to determine the growth of foodborne pathogens in foods. Predictive microbiology refers to the mathematical description of microbial growth or inactivation under several environmental conditions. Temperature is a major factor affecting microbial growth, and temperature can change drastically during processing and storage (Zwietering, Jongenburger, Rombouts, & van't Riet, 1990). Therefore, the effect of temperature on the growth rate of pathogens has been studied extensively and reported in the literature.

The overall goal of the predictive modeling is to predict the population dynamics of microorganisms in food systems under time-varying temperature conditions encountered during processing, storage, and distribution of the food product. Such models are termed as "dynamic models". To develop a dynamic model, models to predict microbial population at various isothermal conditions (primary models) have to be first developed. The secondary model is then developed that describes the effect of environmental conditions on the parameters of the primary model (Whiting, 1995).

Primary Models

Primary models describe the microbial growth under isothermal conditions. Commonly used primary models include the modified Gompertz model (Equation 22.1) and logistic model (Equation 22.2) (Gibson, Bratchell & Roberts, 1987).

$$x(t) = x_o + (x_{max} - x_o)\exp(-\exp(-B(t - M))) \tag{22.1}$$

$$x(t) = x_o + \frac{(x_{max} - x_o)}{1 + \exp(-B(t - M))} \tag{22.2}$$

where $x(t)$ is $\log_{10}$ (CFU/g) of cell concentration at time t; x_o is initial concentration in $\log_{10}$ (CFU/g); B is maximum relative growth rate at M in 1/h; and M is time at which the absolute growth rate is maximum in h. The parameter B can be defined as (McMeekin, Olley, Ross, & Ratkowsky. 1993)

$$B = \frac{r_{max}e}{(y_{max} - y_o)} \tag{22.3}$$

For modified Gompertz model, the specific growth rate r_{max} calculated from the parameter B can be defined as (McMeekin et al., 1993)

$$r_{max} = \frac{x_{max} - x_{min}}{e} \times B \tag{22.4}$$

and for the logistic model, the specific growth rate can be determined by

$$r_{max} = \frac{x_{max} - x_{min}}{4} \times B \tag{22.5}$$

where r_{max} is maximum specific growth rate in terms of $\log_{10}$ cell concentration (1/h).

Secondary Models

Secondary models describe the effects of environmental conditions, such as temperature, pH, a_w, atmosphere (aerobic/anaerobic), and preservatives on the parameters of a primary model, particularly, the maximum growth rate. Most studies focus only on temperature, which is the main environmental condition that changes frequently during storage and distribution of food products.

Modified Ratkowsky Model

A modified Ratkowsky model (Eq. 22.6) was used to analyze the effect of temperature on maximum growth rate (B or μ_{max}) (Zwietering, De Koos, Hasenack, De Wit, & van't Riet, 1991).

$$\mu_{max} = a(T - T_{min})^2.(1 - \exp(b(T - T_{max}))) \tag{22.6}$$

where T is the temperature; T_{min} and T_{max} are the theoretical minimum and maximum temperatures, respectively, beyond which growth is not possible; and a and b are regression constants. Note that T_{min} and T_{max} are model parameters and can be 5–10°C lower or higher than the minimum or maximum temperature at which growth is actually observed. The first term in Eq. (22.6) indicates that the growth rate is proportional to square of the temperature within the range of minimum temperature and optimum temperature of growth. The second exponential term indicates the decrease in growth rate beyond the optimum temperature of growth and until the maximum temperature of growth.

Other secondary models, Arrhenius-based, gamma concept, and cardinal temperature models, have been used to describe the effects of product composition and other parameters on the microbial growth rates.

Dynamic Model

The overall goal of predictive modeling is to develop a model that can predict the microbial population dynamics in food systems for any time–temperature profile. The first step is to develop a primary model for isothermal conditions. Because bacterial growth is sigmoidal under isothermal conditions, sigmoidal-shaped growth models such as the modified Gompertz and the logistic models can be used to fit the growth of microorganisms against time. Then, growth rate from the primary model is modeled as a function of temperature (secondary model). Then, the primary and secondary models are numerically integrated to develop a dynamic model. When these dynamic models are evaluated for dynamic conditions, an incremental value has to be added to the initial inoculum to solve the differential equations (Huang, 2003). Selection of this incremental value has an influence on the lag phase of the microbial growth curve. Huang (2003) developed a computer simulation algorithm that dynamically predicts the growth of *C. perfringens* in cooked ground beef based on the four-parameter modified Gompertz function. However, the incremental value that was added to the initial inoculum was defined in a way to match the observed and predicted values. While these sigmoidal growth models are an excellent means to describe the microbial growth at constant temperatures, they are not suitable for dynamic temperatures.

Baranyi Model—An Innovative Approach

Instead of starting with a standard sigmoidal model such as the logistic model and then extending it to a dynamic model, Baranyi and Roberts (1994) started with a dynamic model.

This model was mathematically expressed as

$$\frac{dx}{dt} = \frac{q}{1+q}\mu_{\max}\left(1 - \frac{x}{x_{\max}}\right)x \tag{22.7}$$

with the initial condition $x = x_0$ at $t = 0$.

$\frac{dx}{dt}$ is the growth rate and $\mu_{\max}$ is the specific growth rate which is equal to growth rate divided by microbial population, $(\frac{dx}{dt})/x$. Thus, the specific growth rate is a product of three terms for three phases, namely lag, exponential growth, and stationary phases. During lag phase, the first term will be close to zero, making the growth rate zero. $q(t)$ is referred to as a bottleneck modeling function (Baranyi & Roberts, 1994), which must reach a certain level to induce growth of the cells. This substance could be, for instance, RNA or other cytoplasmic components such as ribosomes. The middle term, $\mu_{\max}$, is the maximum specific growth rate, while the other two terms will be close to 1 during exponential growth rate. During stationary phase, $x = x_{\max}$, making the third term zero, thereby reducing the growth rate to zero.

The development of the critical substance, q, is assumed to follow first-order kinetics, hence growing exponentially at a constant specific rate, v, as

$$\frac{dq}{dt} = vq \tag{22.8}$$

with the initial condition $q = q_0$ at $t = 0$.

Normally, the specific rate of production of the bottleneck substance, v, is assumed to be equal to that of the bacterial culture. Therefore, Equation (22.8) can be rewritten as (with the same initial condition)

$$\frac{dq}{dt} = \mu_{\max}q \tag{22.9}$$

The initial condition of Equations (22.8 and 22.9) $(q = q_0)$ is a measure of the initial physiological state of the cells. The significance of this model lies in the fact that the adjustment of the cells to a new environment can be characterized by a single parameter, α_0, which is a transformation of q_0 as shown in Equation (22.10).

$$q_0 = \frac{1}{e^{h_0} - 1} = \alpha_0\left(\frac{e^{h_0}}{e^{h_0} - 1}\right) \tag{22.10}$$

The parameter h_0 in Equation (22.10) is simply a numerical stable transformation of q_0 and is considered as the product of the lag time and the maximum specific growth rate, as illustrated in Equation (22.11).

$$h_0 = \mu_{\max}\lambda = \ln\left(1 + \frac{1}{q_0}\right) = -\ln(\alpha_0) \tag{22.11}$$

It has been shown that α_0 (and its related forms q_0 and h_0) is approximately constant in situations where the pre-inoculation histories of the cells are identical (Baranyi & Roberts, 1994; Baranyi, Robinson, Kaloti, & Mackey, 1995; Bellara, McFarlane, Thomas, & Fryer, 2000), within the biokinetic temperature growth range. According to this concept, in a constant environment (i.e., isothermal conditions), the lag time, λ, is inversely proportional to the maximum specific growth rate, $\mu_{\max}$, and the parameter h_0 represents the proportionality constant. With this re-definition of the lag parameter, the Baranyi model offers advantages over other primary models such as the modified versions of the Gompertz function, which generally model the parameters $\mu_{\max}$ and λ independent of each other.

The Baranyi model presented as a set of Equations (22.7 and 22.8) also is commonly found in terms of $y = \ln(x)$ and $Q = \ln(q)$, written as

$$\frac{dy}{dt} = \frac{1}{1 + e^{-Q}}\mu_{\max}\left(1 - e^{(y - y_{\max})}\right) \tag{22.12}$$

$$\frac{dQ}{dt} = \mu_{\max} \tag{22.13}$$

with the initial conditions $y = y_0$ at $t = 0$ and $Q = \ln(q_0)$ at $t = 0$.

It should be noted that $\mu_{\max}$ is a function of temperature, which varies with time. For a special case of isothermal conditions, the above equation can be solved to get a primary model:

$$y(t) = y_0 + \mu_{\max}F(t) - \ln 1 + \frac{e^{\mu_{\max}F(t)} - 1}{e^{(y_{\max} - y_0)}} \tag{22.14}$$

where $F(t) = t + \frac{1}{v}\ln\left(e^{-vt} + e^{-h_0} - e^{(-vt - h_0)}\right)$; $y(t)$ is ln (CFU/g) of cell concentration at time t; y_0 is initial cell concentration in ln (CFU/g) units; $y_{\max}$ is maximum cell concentration in ln (CFU/g) units; $\mu_{\max}$ is maximum specific growth rate in terms of $\log_e$(CFU/g), which is equal to $r_{\max}.\log_e 10$ in 1/h; v is rate of increase of the limiting substrate, assumed to be equal to $\mu_{\max}$; and h_0 is equal to $\mu_{\max}\lambda$ (Baranyi model). Thus, the primary model is a special case of dynamic model. Maximum specific growth rate at each temperature can be estimated and then can be used to develop a secondary model. Then, the dynamic model can be developed by solving the dynamic form of Baranyi model (Eqs. 22.12 and 22.13). Because the Baranyi model is developed

semi-mechanistically rather than empirically, this model performs well for predicting microbial population for dynamic time-varying temperature conditions.

Growth/No Growth Models

These models are also referred as growth/no growth (G/NG) interface models or G/NG boundary models, or "growth boundary" models or simply "growth limits" models. Traditional growth modeling (primary and secondary models) approach is suitable for determining the growth of most foodborne pathogens and spoilage microorganisms. When there are regulatory limits for certain pathogens in particular foods, G/NG boundary models are the most appropriate models. For example, G/NG models are more suitable for *L. monocytogenes* for which there is a zero tolerance in the USA.

The growth or no growth conditions for several combinations of various environmental conditions such as temperature, pH, a_w, and salt content have to be determined. At extreme growth ranges, the growth of microorganisms is very erratic and makes the development of these models more complex. Thus, the probability of growth is modeled due to uncertainties involved in bacterial growth under sub-optimal conditions. It is important to note that the probability of growth is strongly time dependent. Therefore, these models have to be defined for certain time period, which is typically the storage life of the product.

Logistic models are commonly used to describe the probability of growth for given conditions. Logistic models are defined as (Ratkowsky, 2002)

$$log\left(\frac{p}{1-p}\right) = \text{logit[P]} = b_0 + b_1 X_T + b_2 X_{pH} + b_3 X_{aw} + b_4 X_{NO_2}$$

or

$$p(x) = \frac{e^{b_0 + b_1 X_T + b_2 X_{pH} + b_3 X_{aw} + b_4 X_{NO_2}}}{1 + e^{b_0 + b_1 X_T + b_2 X_{pH} + b_3 X_{aw} + b_4 X_{NO_2}}}$$

where $X_T = \ln(T - T_{\min})$, $X_{\text{pH}} = \ln(\text{pH} - \text{pH}_{\min})$, $X_{\text{aw}} = \ln(a_w - a_{w\min})$, $X_{NO_2} = \ln(NO_2 - \max - NO_2)$ are environmental variables such as temperature, pH, a_w, or salt content and P is the probability of observing growth (a value of 1).

The logit(P) can be interpreted as log of ratio of odds for growth and odds for no growth.

$$log\left(\frac{P}{1-P}\right) = log\left(\frac{probability\,of\,growth}{1-probability\,of\,growth}\right) = log\left(\frac{probability\,of\,growth}{1-probability\,of\,no\,growth}\right)$$
$$= log(odds\,ratio)$$

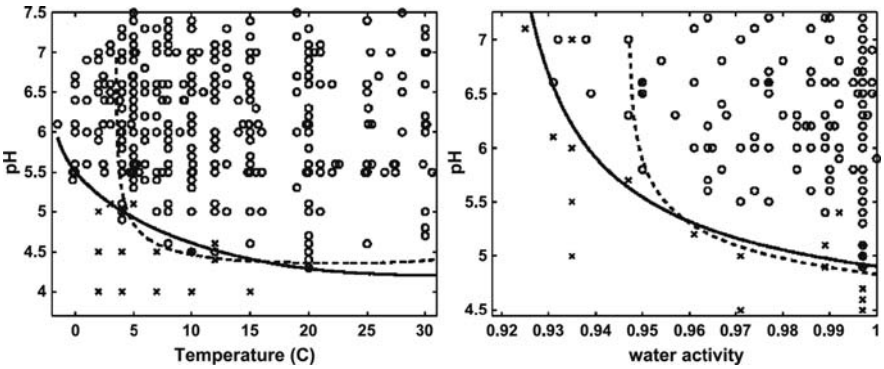

Fig. 22.1 Growth/no growth interface of *L. monocytogenes* as a function of temperature and pH and water activity and pH (Le Marc, Pin & Baranyi, 2005)

If the probability of growth (P) is 0.75, then the probability of no growth is 0.25. Then, the odds of growth are 3 (0.75/0.25). Thus, there is three times more probability to observe growth than no growth. The log (odds) is then modeled as a function of logarithm of environmental variables. One of the major challenges in developing G/NG boundary models is the large number of data points necessary, which requires significant time and resources to generate the data. Marc, Pin, and Baranyi (2005) used the ComBase database to collect large amount of growth/no growth data for several pathogens and developed G/NG models. Figure 22.1 shows the G/NG model for *L. monocytogenes*. A contour boundary can be drawn for each probability level of growth in a multidimensional environmental space.

Predictive Models for Pathogens of Significance in Cooked Meats

The microbial safety of foods has traditionally been determined by conducting experiments (microbial challenge studies) to mimic the product manufacturing process and the final product characteristics with ingredients inoculated with a pathogen of concern. However, the diversity of the products, ingredients, and manufacturing processes renders this process to be tedious and applicable to only the specific product–process combination. Modifications in any of the ingredients or the process can have significant impact on the survival and/or growth of the microorganisms, especially the foodborne pathogens. The availability of personal computers at affordable prices and the accessibility of such data through the Internet have made the predictive models a necessary tool for evaluation of safety of products and manufacturing processes. Today, these predictive microbial models are routinely used by the industry personnel as well as the regulatory agencies to provide an insight into the microbiological safety of the food manufacturing processes and the resulting food products.

C. botulinum *Predictive Models for Cooked Meats*

A brief overview of the models for *C. botulinum* growth was discussed by McClure, Cole, and Smelt (1994). The earliest models developed for *C. botulinum* were for predicting the survival of the spores during thermal processing (canning). Subsequent models for probability of growth or time to toxin production have been reported in the literature. The factors that have been used for preserving foods at risk for *C. botulinum* growth and toxin production include pH, NaCl, sodium nitrite, and sorbate among others. Predictive models describe the effects of these factors in a variety of food matrices such as cheese spreads, pork slurry (Lindström et al., 2001), and *sous vide* beef and *sous vide* pork (Hyytiä-Trees et al., 2000).

Genigeorgis, Meng, and Baker (1991) used a two-step approach, using regression analysis to model lag phase, and subsequently incorporated these into secondary equations to express or predict the probability of toxin production, resulting from *C. botulinum* growth in turkey homogenate. A more user friendly approach to modeling, using kinetic models (primary and secondary models) with a variety of parameters a_w, pH, NaCl, and nitrite utilizing meat products, would be more appropriate as models generated using microbiological media may not provide "realistic" estimates of *C. botulinum* growth.

Polynomial expressions that incorporated the environmental variables (pH, a_w, NaCl, etc.) were used to develop probability models (logistic) to estimate the probability of toxin formation by *C. botulinum* (Roberts, Gibson & Robinson, 1981). Subsequently, Lindroth and Genigeorgis (1986) developed a probability model assuming germination, growth initiation, and toxin production from a single spore, using a similar expression.

Primary–secondary models use (i) either a kinetic model to describe lag time and growth of the organism or a probability model to predict the chance of toxin formation over time and (ii) another model to predict the effect of environmental factors on the parameters of the first model (Schaffner, Ross, & Montville, 1998).

Gibson et al. (1987) developed a kinetic model for the growth of *C. botulinum* type A in pasteurized pork slurry by using logistic and Gompertz functions. The relationship between the time to reach the maximum rate of growth and incubation temperature and sodium chloride concentration was described graphically.

Whiting and Call (1993) used nonlinear regression to estimate the parameters of a primary model for probability of growth at a given time and then used polynomial expressions containing experimental variables to predict the parameters of the primary model. This approach was expanded to develop a model for non-proteolytic type B *C. botulinum*, where inoculum size and time-to-toxicity confidence intervals were also included in the model (Whiting & Oriente, 1997).

While the modeling approaches for probability models and kinetic models are different, both the approaches basically predict the ability of the organism to grow and, in case of growth, subsequent production of toxin (Schaffner et al., 1998). Other approaches used to model *C. botulinum* behavior include waiting time modeling (Ter Steeg & Cuppers, 1995) to develop expressions for the effect of environmental parameters on time for a specific event (toxin production, turbidity development, or growth of the organism). Waiting time models can be used whenever the time to the occurrence of some event is the variable of interest. In the case of the time-to-toxicity data, this is the time from the beginning of an experiment until a tube is identified as positive. Schaffner et al. (1998) stated that waiting time models can be easily developed using currently available statistical analysis software; the models are flexible and are simple to interpret (Fig. 22.2).

Rogers and Montville (1994) used linear regression to model the factors that influence the ability of nisin to inhibit *C. botulinum* in a model food system. Subsequently, Schaffner et al. (1998) used the waiting time modeling approach to analyze the combined effects of temperature, pH, carbohydrate, protein, and lipid on the time-to-toxicity of *C. botulinum* using data from Rogers and Montville (1994). Fernandez, Baranyi, and Peck (2001) developed a model (quadratic, multivariate response surface) to predict the growth of non-proteolytic *C. botulinum* in a model system (PYGS broth) at various pH, NaCl, temperature, and CO_2 concentrations (modified atmospheres).

While there were several studies indicating the time to toxin production or turbidity for *C. botulinum*, the minimum populations required to produce toxin have not been reported. Review of Elliott and Schaffner (2001) indicates that the *C. botulinum* populations were $\geq$5.0 log CFU/ml before toxin was detected

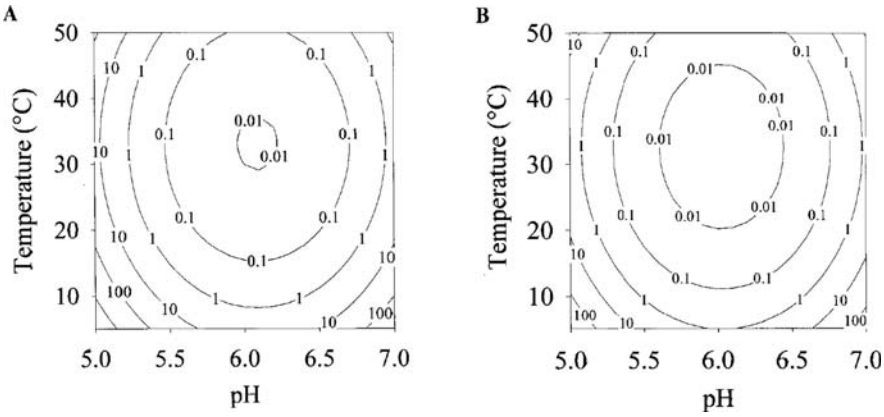

Fig. 22.2 Predicted time-to-toxicity (in days) of *C. botulinum* in a model system as influenced by macronutrients, incubation temperature, and pH. *Contour lines* represent the predicted time at which the probability of toxicity is one in a million (Schaffner, Ross, & Montville, 1998)

in TPGY broth containing NaCl (0.25 or 1.75%), at pH 5.75 or 6.5 and temperature of either 7 or 13°C. It would not be prudent to allow the growth of *C. botulinum* regardless of the ability of the organism to produce toxin in the product in question. The USDA-FSIS stabilization requirements specify no growth of *C. botulinum* during cooling of the meat and poultry products (USDA-FSIS, 1999). During cooling of meat and poultry products, process deviations can occur, resulting in abusive cooling rates (beyond the safe harbors), either at the higher temperature range (54.4–26.7°C) or the lower temperature range (26.7–4.4°C). In terms of *C. botulinum* spore germination and outgrowth, the potential for either proteolytic and non-proteolytic types or both should be evaluated based on the product temperature (range) where the deviation occurred and the cooling profile of the product.

Hyytiä-Trees et al. (2000) evaluated the performance of the "tertiary" models for non-proteolytic *C. botulinum* (UK Food MicroModel; FMM and USDA-ARS Pathogen Modeling Program) growth in *sous vide*-processed products and reported significant variation between the safe storage time predictions from the software and the challenge study. The authors ascribed the poor agreement between the predictions from the software and the challenge study to the limited number of controlling factors in the models (Hyytiä-Trees et al., 2000). They stated that with similar types of products (meat-based *sous vide*) that rely on refrigeration to inhibit the growth of non-proteolytic *C. botulinum*, predictive models should not be used and that the safety evaluation be based on challenge studies.

C. perfringens *Predictive Models for Cooked Meats*

Several models have been developed and are currently available to predict *C. perfringens* germination and outgrowth in meat and poultry products. The most widely used "tertiary" predictive models (software/graphic user interface) are the USDA Agricultural Research Service (USDA-ARS) Pathogen Modeling Program (PMP) and the Perfringens Predictor. These programs have an easy interface, where the user could upload the temperature profile of the product and predict the potential germination and outgrowth of *C. perfringens*. While the PMP was developed using isothermal growth data for *C. perfringens* using microbiological media, recent updates to the software include models developed using meat systems. The underlying models used in Perfringens Predictor were collected from literature and was used to develop a dynamic model that allows the user to specify the temperature profile of the product to evaluate potential *C. perfringens* spore germination and outgrowth.

One of the first approaches to predictive modeling of *C. perfringens* was by Labbe and Huang (1995) using laboratory media (fluid thioglycollate medium), media supplemented with beef, and autoclaved ground beef matrices. As reported in a previous research (Willardsen, Busta, & Allen, 1979), faster growth rates were observed in autoclaved ground beef compared to laboratory media.

Subsequently, Juneja, Marmer, Phillips, and Palumbo (1996) developed a predictive model for vegetative growth of *C. perfringens* that incorporated interactive effects of temperature (12–42°C), product pH (5.5–7), sodium chloride (0–3%), and sodium pyrophosphate (0–0.3%) using a model system (trypticase–peptone–glucose–yeast extract broth). The maximal growth rate was observed at 42°C, pH 6.25, with a GT of 12 min and a lag phase duration of 2.27 h. Interactions between the ingredients and product pH on *C. perfringens* growth were observed. This report only provided information on *C. perfringens* growth at isothermal temperatures, and a secondary model to explain *C. perfringens* growth with changes in temperatures (dynamic) over time was not developed.

Further, RTE meat and poultry products are currently formulated to contain salt, phosphates, curing agent (sodium nitrite), a reducing agent (sodium erythorbate), and quite often antimicrobial ingredients such as organic acid salts (sodium or potassium salts of lactic or citric acids). These ingredients have been shown to affect the germination and outgrowth of *C. perfringens* in meat systems. However, modeling of all these parameters, taking into consideration the variation in concentrations used by RTE meat and poultry processors, may be a daunting task. However, judicious application of predictive models that estimate the potential germination and outgrowth of *C. perfringens* under worst-case scenario with some information derived from challenge studies evaluating the effects of antimicrobial agents can provide reasonable assurance on the safety of the resulting products.

Limitations of the earlier models include the use of laboratory media, especially since *C. perfringens* can grow faster in meat systems compared to laboratory media (Willardsen et al., 1979). In a subsequent report, Juneja, Whiting, Marks, and Snyder (1999) described a secondary model for growth of *C. perfringens* from heat-activated spores during cooling using a meat system (autoclaved ground beef). The limitations of this model have been highlighted by Smith and Schaffner (2004), indicating that the exponential growth rates (EGR) were responsible for the under-prediction of *C. perfringens* growth rather than the germination, outgrowth, and lag phase (GOL). The authors report that the model performed relatively well (fail safe) when low (<1 log CFU/ml) or high (>3 log CFU/ml) growth was observed (increases) during exponential cooling. However, the model consistently under-predicted growth at intermediate observed increases (1–3 log CFU/ml) as well as in trials using two different rates of exponential cooling.

In an effort to simplify this process, Huang (2002) described the outgrowth of heat-activated spores of *C. perfringens* in cooked beef and developed a multiple linear model. The growth curves at various temperatures were generated and fitted to the Gompertz equation and a modified multiple linear model. The model consisted of five linear segments to describe the sigmoidal growth of *C. perfringens* for each temperature. The growth curve was divided into five linear segments described as lag, first transitional, exponential, second

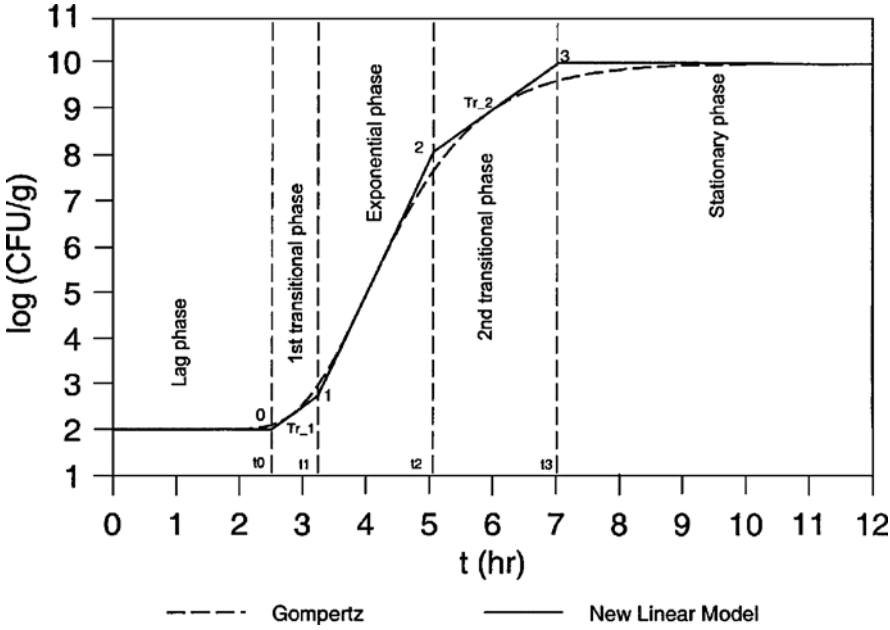

Fig. 22.3 Differentiation of microbial growth process info five segments: lag, first transitional, exponential, second transitional, and stationary phases (Adapted from Huang, 2002)

transitional, and stationary phases (Fig. 22.3). This allowed the author to derive lag phase duration parameters as a linear function of the traditional lag phase duration calculated from the Gompertz equation. This in turn permits three-segment linear models to be used to generate five-segment linear growth curves with no need to solve mathematical functions. For the linear models, the mean growth rates observed in the transitional phases were considered the same. The primary models were then fitted to a square root function to determine the effect of temperature on growth parameters. Huang (2002) concluded that the linear method accurately described the sigmoidal shape of growth curves and provided similar parameters for secondary modeling as the Gompertz function.

Recent research includes a model for cured pork ham using the Baranyi model to determine growth kinetic parameters of isothermal growth curves and the square root Ratkowsky model to represent the exponential growth rates as a function of temperature and the Runge–Kutta procedure to solve the numerical functions (Amezquita, Wang, Weller, Thippareddi, & Burson, 2005). Similar methodologies were also used to develop predictive models for cured and non-cured roast beef, cured and non-cured ground pork, and cured and non-cured ground turkey.

Research is needed to determine the interactive effects of other ingredients in the formulation of meat products, as the ingredients such as curing salts, phosphates, and salts of organic acids affect the growth of this organism. Formulating buffered sodium citrate (1.3%), buffered sodium citrate supplemented with sodium diacetate (1.3%), as well as a mixture of sodium lactate and potassium lactate (2.5%), and a mixture of sodium lactate and sodium diacetate (2.5%; 6:4 mixture) were shown to be sufficient to completely inhibit the growth of *C. perfringens* during extended cooling of injected meat products (Thippareddi, Juneja, Phebus, Marsden, & Kastner, 2003).

Since the issue of germination and outgrowth of *C. perfringens* spores is time dependent, and during cooling a continuously varying temperature conditions exist, the models should be able to predict the germination and outgrowth of *C. perfringens* spores under those conditions. As has been reported, the germination process, outgrowth, or both can be affected by antimicrobial ingredients present in the meat and poultry products. Further, RTE meat and poultry processors may encounter cooling deviations, either due to power failure or refrigeration system failure, resulting in non-continuous chilling rates. In such circumstances, the models should be robust enough to be able to accurately predict the potential germination and outgrowth of *C. perfringens* spores in the particular product of concern. Amezquita et al. (2005) developed a finite element heat diffusion model to predict the temperature of meat product (ham) and integrated it with *C. perfringens* growth model. Such models allow processors to evaluate the adequacy of their cooling systems or design products with dimensions that allow proper cooling of the products to minimize the risk of *C. perfringens* germination and outgrowth (Fig. 22.4).

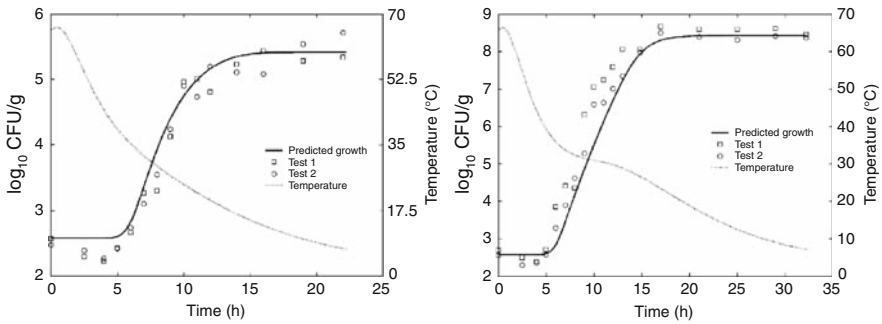

Fig. 22.4 Comparison of predicted and observed *C. perfringens* growth during cooling of boneless cooked cured ham, when a deviation from FSIS compliance guidelines occurs at 1.8 h into cooling. The simulated deviation is caused by unexpected equipment failure or electrical outage for a total downtime of 1 h. Cooling time from 54.4 to 26.6°C is 6.6 h and from 26.6 to 7.2°C is 13.2 h (Amezquita et al., 2005)

B. cereus *Predictive Models for Cooked Meats*

Olmez and Aran (2005) developed models to describe the growth kinetic parameters (lag phase duration and growth rate) of *B. cereus* as a function of temperature (8, 15, 26, and 32°C), pH (5.3, 5.8, 6.3, 6.8, and 7.3), and sodium lactate (0, 200, 400, and 600 mM) and sodium chloride (85, 342, and 600 mM) concentrations. Microbiological media (brain heart infusion) was used to develop the model, and microbial growth was determined by measuring the optical density changes in the medium. The primary model was developed using Gompertz function, while polynomial equations (secondary model) were used to describe the lag and generation times. The authors reported that temperature, pH, sodium lactate, and sodium chloride had a significant effect on the growth of *B. cereus* and should be considered in predicting the pathogen growth. While the model performed well (mean absolute relative error of 25.6% and median relative error of 16.7%), it should be noted that the growth kinetics can be significantly different in foods, and also if the organisms are present in raw material and the ingredients used for the manufacture of the cooked meats, it would be in spore form. The germination, outgrowth, and lag phase duration could be different when the organism is growing from a spore state rather than from vegetative form.

In an earlier report (Choma et al., 2000), growth kinetics of *B. cereus* were determined at temperatures between 5 and 40°C in courgette broth and rich medium (J broth). Arrhenius equation (primary model) and Ratkowsky's model (secondary model) were used to fit the experimental data. In addition, a few other studies (Quintavalla & Parolari, 1993; Chorin, Thuault, Cleret, & Bourgeois, 1997; Baker, & Griffiths, 1993; Benedict, Partridge, Wells, & Buchanan, 1993) evaluated the growth of *B. cereus* in microbiological media, but not in food products. Predictive models for *B. cereus* growth developed and/or validated in food products rather than microbiological media would be of interest for food processors as well as regulatory agencies.

L. monocytogenes *Predictive Models for Cooked Meats*

The USDA-FSIS requires RTE meat and poultry processors to minimize the risk of *L. monocytogenes* in the RTE processing environment and the RTE meat and poultry products. The USDA-FSIS final rule on "*Listeria monocytogenes* Contamination of the Ready-to-Eat Meat and Poultry Products" has identified three alternatives for RTE meat and poultry processors to address this pathogen in their food safety programs. RTE meat and poultry processors can control *L. monocytogenes* by (i) using a post-lethality treatment and an antimicrobial agent or process (Alternative 1); (ii) using either a post-lethality treatment or an antimicrobial agent or process (Alternative 2); or (iii) control of *L. monocytogenes* in the post-lethality processing environment through

sanitation procedures only (Alternative 3). For Alternatives 1 and 2, the RTE processors can use antimicrobial agents to minimize the risk of *L. monocytogenes* growth in their products, resulting in a lower risk of foodborne illness (USDA-FSIS, 2003).

The antimicrobial agents commonly used in the RTE meat and poultry industry in the USA currently include mixtures of organic acid salts (sodium or potassium salts of lactic acid or citric acid in combination with sodium diacetate). The USDA-FSIS defined antimicrobial agent as a substance in, or added to, a RTE product that has the effect of either reducing or eliminating a microorganism, including a pathogen such as *L. monocytogenes*, or that can limit or suppress the growth of *L. monocytogenes*. The antimicrobial should be effective throughout the shelf life of the product (USDA-FSIS, 2000). The antimicrobial can be added to the product during formulation, to the finished product, or to the packaging material to inhibit growth of *L. monocytogenes* in the post-lethality exposed product during its refrigerated shelf life.

While seafood and dairy products have also been identified as risk factors in foodborne illness due to *L. monocytogenes*, RTE meat and poultry products have been the focus for development of most predictive models in the USA. The application of predictive models for *L. monocytogenes* has been to evaluate the potential shelf life (safety based) the RTE meat and poultry processor can declare on the label to meet the regulatory requirements. As such, models that were developed using microbiological media are of limited use in this context. A good example of a model that the industry routinely uses is the Opti.Form model, developed and marketed by Purac America (Lincolnshire, IL). This tertiary model allows the user to define the product specifications such as moisture and salt content and the expected "time to growth" (time to 1 log increase) or alternatively the shelf life (safety based) is predicted based on the concentrations of lactate and/or diacetate. Most RTE meat processors use this model as supporting documentation for their HACCP plans and as justification for regulatory purposes.

The initial iteration of the model was based on the study by Seman, Borger, Meyer, Hall, and Milkowski (2002); subsequent study (Legan, Seman, Milkowski, Hirschey, & Vandeven, 2004) incorporated the option to include whether the product contained sodium nitrite (cure) or not. The model was developed using a generalized regression approach, alternatively termed "survival analysis" or "reliability analysis" within the biomedical and engineering fields of study. Basically, the model is a "boundary" model that defines the time to reach 1 log growth of *L. monocytogenes* in a product (based on its composition). The use of least squares regression to develop such models has the disadvantage that the points where no growth was observed cannot be included. The study highlights the significance of product composition (moisture, salt, and sodium nitrite) on *L. monocytogenes* growth and provides the user to predict the potential *L. monocytogenes* growth for a specific product, rather than providing a "conservative" estimate of the growth. This ability to tailor the predictions to their specific product is very useful for the processors in designing

their product formulations to attain a specific "shelf life" based on microbiological safety. A similar approach was adopted by Seman, Quickert, Borger, and Meyer (2008) to predict *L. monocytogenes* growth on RTE meat and poultry products containing sodium benzoate as an antimicrobial agent.

However, a drawback of the model is that the experimental design included only one specific storage temperature (4°C) to predict *L. monocytogenes* growth, and the model does not allow or predict growth at other refrigeration temperatures. While it is desirous to maintain a specific temperature, in reality, it is impossible to achieve that throughout the cold chain, and especially at the consumer stage. Further, the product temperature fluctuations occur throughout the cold chain and the models that can predict *L. monocytogenes* growth during those fluctuating or constantly varying temperatures (dynamic) such as those for *C. perfringens* will be of more utility for the processors as well as regulators in evaluating the realistic growth potential of the organism in the marketplace. The model design adopted by Seman et al. (2002) and Legan et al. (2004) does not render itself useful in such circumstances.

In a recent study, Monsalve (2008) developed a model to predict the growth of *L. monocytogenes* on RTE roast beef and turkey using the traditional approach of obtaining growth parameters under isothermal conditions and subsequently developed the dynamic model (Baranyi). While the model does not include a range of moisture and salt concentrations in the products, Monsalve incorporated various temperatures, allowing for prediction of the potential *L. monocytogenes* growth at a minimal salt concentration (2%) using a traditional product formulation, with different concentrations of the antimicrobial (buffered sodium citrate containing sodium diacetate). This model allows a "conservative" prediction of potential *L. monocytogenes* growth in the products at different concentrations of the antimicrobial under dynamic temperature conditions (Fig. 22.5). Such models will be very helpful for

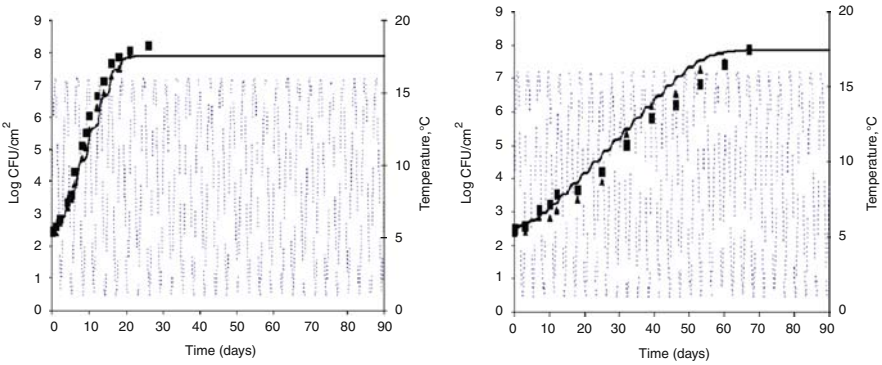

Fig. 22.5 Predicted and observed growth of *L. monocytogenes* on cured, RTE turkey hams containing buffered sodium citrate (BSC) and sodium diacetate (SD); BSC + SD (0%) and BSC + SD (1%) at constantly varying temperatures (Monsalve, 2008)

processors to evaluate the safety of the product under existing distribution channels or the potential risk of introducing a RTE meat and poultry product into new marketing channel by measuring and incorporating the temperature profiles of the distribution channels (cold chain).

While other models have been developed and published in the literature, they do not serve the needs of the cooked meats (RTE) processors or the risk managers the tool to evaluate the potential risk of *L. monocytogenes* growth during the shelf life of the product and through to the consumption stage.

Conclusions

Significant progress in development and application of predictive models in food microbiology has been made since the 1980 s. Today, predictive models are widely used in the cooked meat industry to evaluate the shelf life (safety based), potential risk of pathogen growth, as well as in design of cooked meat product formulations to minimize the risk of pathogen growth. These predictive models have provided the industry the means to manage the risks of foodborne pathogens throughout the cold chain. However, caution should be exercised in the application of such models to specific cooked products as the product formulations and manufacturing processes can significantly affect the propriety and applicability of the predictive models.

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Chapter 23
Microbiological Quantitative Risk Assessment

Silvia Dominguez and Donald W. Schaffner

Introduction

The meat and poultry industry faces ongoing challenges due to the natural association of pathogens of concern (e.g., *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* O157:H7) with a variety of domesticated food animals. In addition, pathogens such as *Listeria monocytogenes* pose a significant cross-contamination risk during further meat and poultry processing, distribution, and storage. Furthermore, the meat and poultry industries are constantly changing with the addition of new products, use of new raw materials, and targeting of new consumer populations, each of which may give rise to potential new risks. National and international regulations are increasingly using a "risk-based" approach to food safety (where the regulatory focus is driven by the magnitude of the risk), so risk assessment is becoming a valuable tool to systematically organize and evaluate the potential public health risk posed by food processing operations.

In this chapter, representative quantitative microbiological risk assessments developed by independent researchers, national regulatory agencies, and international agencies will be discussed in an attempt to exemplify different applications of this tool in its current state of development, as well as highlight potential future uses.

Quantitative microbial risk assessment (QMRA) is a methodology used to organize and analyze relevant data in order to estimate the public health consequences associated with microbiological risk. Historically it has been used to assess the risk posed by both contaminated food and water, although here we will focus on only food risk, particularly the risk posed by fresh and processed meat. QMRA considers some or all of the various stages in the food production process, and the main outcome of QMRA is traditionally defined as

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the estimated probability of illness from the consumption of the food product under study. However, this methodology has also been successfully applied to provide valuable information on the microbial consequences of specific processing steps on the food production chain, as well as during handling by the consumer, in particular, those that contribute to an increased risk of foodborne illness (Cassin, Lammerding, Todd, Ross, & McColl, 1998). Once the risk model is developed, different scenarios can be analyzed by varying the inputs of particular modules. Individual process steps, as well as risk mitigation strategies, can be evaluated under this scheme to determine their impact on the overall risk (Vanderlinde, 1998); the results of these risk assessment simulations may provide scientific basis for the evaluation of risk management alternatives. In a QMRA, the identification of factors which most significantly contribute to risk is often referred to as sensitivity analysis (Cassin et al., 1998). Examples of risk mitigation strategies that may be considered within a QMRA for meat products include the reduction of on-farm prevalence of the pathogen of concern, reduction in storage temperatures, and the inclusion of a decontamination step, among others.

Monte Carlo simulation is currently the most widely used technique for conducting microbial risk assessments. This methodology uses a stochastic approach, where key factors in the model are represented by distributions, and a set of output values in the form of a distribution is generated as a result of multiple iterations. Thus, input data in the form of distributions of probability (as opposed to discrete values) – for example, for the prevalence and levels of pathogen in a carcass or for thermal inactivation during a cooking step – are combined to generate an estimated probability of illness which is also represented as a distribution. Because high-risk scenarios often arise from outlying data points rather than average results (Whiting, 1997), Monte Carlo simulation has the potential to provide a more realistic estimation of risk compared to a strictly deterministic approach. In addition, taking into account the variability described by a frequency distribution produces a more realistic assessment of risk than one based on a sole discrete value, such as the mean or worst case, at each step modeled (Brown, Davies, Billon, Adair, & McClure, 1998). Unfortunately, many previously published studies present microbiological data as discrete values (e.g., the mean log CFU/g) rather than distributions, which limits its use for QMRA (Nauta, van der Fels-Klerx, & Havelaar, 2005).

Risk assessment in general and QMRA in particular is often described as consisting of four stages: (1) hazard identification, in which the pathogenic microorganisms potentially present in the food product are identified; (2) hazard characterization, which describes the adverse health effects associated with the microorganism if consumed; (3) exposure assessment, which provides an estimated frequency of consumption of the food in study, and the probable number of microorganisms per serving; and (4) risk characterization, where hazard characterization and exposure assessment are integrated to provide an estimated risk of infection associated with the consumption of the food

product. For meat products, a farm-to-fork approach would, in theory, provide the most comprehensive estimation of risk; however, as will be further discussed in this chapter, the lack of crucial data makes this task difficult. Furthermore, the scope of the QMRA should be decided based on the purpose of the assessment and the questions it is intended to answer; in some cases a farm-to-fork approach may not be appropriate (Kelly et al., 2003). In general terms, the distribution of pathogens in the raw material, changes in pathogen population during manufacture, distribution and storage as well as during preparation at home need to be integrated with a dose–response model to estimate the probability of illness. In-farm contamination of animals may be included in the QMRA; because this is a very complex phenomenon, it is usually modeled separately and the results used as inputs for a subsequent processing and consumption QMRA.

The safety of meat and processed meat products has been in recent years the focus of several risk assessments. As discussed elsewhere in this book, a number of pathogenic microorganisms are frequently associated with meat and poultry products. Commonly recognized examples include *Salmonella* and *C. jejuni* in poultry, *E. coli* O157:H7 in beef, and *L. monocytogenes* in deli meats. A large quantity of both surveillance and epidemiological data supports the association of these pathogens with meat products. The public health risk posed by different pathogens/products has also been assessed using QMRA such as *Salmonella* DT104 in dry-cured pork sausage, fluoroquinolone-resistant *Campylobacter* spp. in broilers, or *L. monocytogenes* in fermented meat products, among others.

Peer-Reviewed QMRAs

Since the mid-1990s peer-reviewed studies presenting QMRAs, particularly those related to meat and poultry products, have increased significantly. A number of these studies demonstrated the applicability of this methodology as a decision-making tool for different food safety issues. Significant data gaps have also been identified, thus encouraging the collection of new data crucial for the development of more accurate risk assessments. Certainly the adoption of the Sanitary and Phytosanitary agreement (WTO, 1995) by the World Trade Organization member states in 1995, which requires that all food safety regulatory measures should be based on scientific risk assessments, was a major global event that encouraged research in this area.

Some of these peer-reviewed studies describing QMRAs based on Monte Carlo simulation techniques will be further discussed in this chapter (Table 23.1). These studies were selected in order to exemplify a wide range of QMRA applications for the meat industry.

Whiting (1997) demonstrated the use of previously published predictive models as input sources complementing the risk assessment methodology in

Table 23.1 Peer-reviewed quantitative microbial risk assessments for meat and processed meat

Pathogen	Meat/Poultry Product	Scope	Reference
Salmonella	Chicken patty	Retail to consumption	Whiting, 1997
Salmonella	Whole chicken	Plant exit to consumption	Oscar, 1998, 2004
Salmonella	Turkey "cordon bleu"	Consumption at catering establishment	Bemrah et al., 2002
Salmonella DT104	Dry-cured pork sausages	Prevalence in pork to prevalence in finished product	Alban et al., 2002
C. jejuni, *Fluoroquinolone-resistant C. jejuni*	Beef and ground beef	Retail to consumption	Anderson et al., 2001
Campylobacter	Birds	Farm	Hartnett, 2001
Campylobacter	Chicken	Farm to consumption	Kelly et al., 2003
Campylobacter	Chicken	Slaughter to consumption	Rosenquist et al., 2003
Campylobacter	Poultry-based meat preparations	Retail to consumption	Uyttendaele et al., 2006
E. coli O157:H7	Ground beef hamburgers	Processing to consumption	Cassin et al., 1998
L. monocytogenes	Ham	Retail to consumption	Perez-Rodriguez et al., 2006
Not specific	Chicken	Processing (slaughter to chilling)	Nauta et al., 2005

an example for the probability of infection from *Salmonella* in a cooked poultry patty. Though not specific for*Salmonella* in poultry patties, it demonstrated that models developed on laboratory media or eggs could serve as surrogates for the growth and inactivation modules simulated using Monte Carlo methodology. The ability of QMRA to provide information on which factors have a major influence on the final probability of infection was also demonstrated. In this QMRA, higher initial populations and slight variations in cooking temperature had the most significant impact on the final probability of illness.

Oscar (1998) developed a QMRA model for the risk of salmonellosis associated with the consumption of whole chickens. The results of this QMRA showed that chickens highly contaminated at the point of exit from the plant did not necessarily pose greater risk of salmonellosis than less contaminated chickens. Less contaminated chickens were found to have a larger impact on the risk of salmonellosis when they were temperature abused or undercooked. The input settings for this QMRA were empirically derived and the use of previously published data was limited. Oscar (2004) improved the 1998 model with the use

of existing data and predictive models, as well as the inclusion of a cross-contamination module. Though cross-contamination is a complex and poorly understood event, this study demonstrated that it is possible for it to be modeled using food handling surveys' data along with published studies with surrogate bacteria to provide a more realistic QMRA. Also, it was demonstrated again that surrogate models, in this case for the growth of *Salmonella* in autoclaved chicken and for thermal inactivation in chicken patties, may provide valid inputs for QMRA.

Cassin et al. (1998) developed a QMRA for *E. coli* O157:H7 in a particular ground beef hamburger manufacturing scenario. In this QMRA, risk was modeled from the contamination of beef carcasses at the beginning of the production chain to the consumption of hamburgers cooked in the home. Detailed considerations were provided for the assessment of risk through the different steps included in the scope of this QMRA, including concentration and prevalence in carcass, processing, post-processing microbial growth, and thermal inactivation and consumption. As expected from the use of Monte Carlo techniques, the results of this QMRA are expressed as a distribution for the probability of illness. This distribution includes a range of risk associated with persons who eat rare hamburgers and consumers potentially more sensitive to infection. In the sensitivity analysis, improved compliance in reducing storage temperatures was predicted to significantly reduce the incidence of illness. A consumer information program to increase cooking temperatures had a lower impact in reducing the probability of illness, due to a predicted reduced level of compliance. In an attempt to validate the results of the QMRA, the average value of probability of illness obtained was compared to the estimated number of annual illnesses attributed to *E. coli* O157:H7 in the United States. After taking into consideration which proportion of this total illness data may be attributed only to consumption of hamburgers, the QMRA estimated a higher probability of illness. As the authors explain, the home cooking scenario modeled in this QMRA might be expected to have a risk greater than that associated with the consumption of frozen patty hamburgers cooked in restaurants.

Hoornstra and Notermans (2001) provided an interesting demonstration of the applicability of QMRA as a tool to (1) set a microbiological criterion and (2) determine if a process meets a microbiological criterion. In the first case, the objective of the risk assessment was to determine if a criterion should be set for the reduction of *E. coli* O157:H7 during the production of raw fermented sausages. Experimental data on the reduction of *E. coli* O157:H7 numbers during the production of raw fermented sausages were collected for the purpose of this study and integrated with the data of Cassin et al. (1998). Using Monte Carlo simulations, the risk assessment demonstrated that through the different stages of the production of raw fermented sausages, a 2- to 3-log reduction was achieved and the prevalence of positive samples was also continuously decreased. According to US Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) regulations, a final product sausage should

contain less than one *E. coli* O157:H7 CFU/100 g and the overall process must provide a 5-log reduction in *E. coli* O157:H7 concentration. These regulations are based on a worst-case scenario that assumes initial contamination of a beef carcass with 3-log CFU of *E. coli* O157:H7/g. This would imply that an extra inactivation treatment such as thermal inactivation may be required in the production of fermented sausages to achieve safe levels of *E. coli* O157:H7. However, Hoornstra and Notermans (2001), using a Monte Carlo simulation approach and considering a more realistic distribution of *E. coli* O157:H7 in a beef carcass according to surveys from the Netherlands, demonstrated that the risk of a raw fermented sausage actually being positive for *E. coli* O157:H7 with pathogen levels above the permitted limit is only 0.002%. In the second case, the objective of the risk assessment was to determine how a food company producing a pasteurized meat product could achieve a 5D reduction of *E. coli* O157:H7. By using a Monte Carlo simulation approach, the results of different process parameters were modeled as probability distributions of the inactivation of *E. coli* O157:H7, and thus optimum pasteurization parameters were proposed, achieving >5D reduction.

Anderson, Woo, and Crawford (2001) applied Monte Carlo simulation to develop a QMRA to analyze the potential public health risk from *C. jejuni* and fluoroquinolone (FQ)-resistant *C. jejuni* associated with the consumption of beef and ground beef in the United States. The model's scope included steps from prevalence in retail beef to consumption at home, though cross-contamination was not modeled. The prevalence in cattle of FQ-resistant *C. jejuni* was derived from human clinical sources due to the lack of data from animal sources. The main challenge addressed in this study was to assess the relationship between levels of FQ resistance in animals and the prevalence of resistance in humans. For modeling purposes, data for the number of years of use of FQ in cattle in different countries were correlated with the corresponding reported number of infected individuals who become ill with FQ-resistant *C. jejuni*. For non FQ-resistant *C. jejuni*, the QMRA results provided a slight underestimation of risk when compared against CDC estimates for illness attributed to *C. jejuni* in the United States. For FQ-resistant *C. jejuni*, the model results were presented as the number of individuals infected with *C. jejuni* who will not respond to FQ treatment as a function of the number of years of FQ use in cattle.

Kelly et al. (2003) constructed a QMRA for *Campylobacter* infection in Great Britain associated with the consumption of broiler products. As described in Hartnett, Kelly, Gettinby, and Wooldridge (2002), this risk assessment takes a farm-to-fork approach. The previously developed risk assessment by Hartnett (2001) was incorporated into this QMRA in the rearing module to estimate the probability that a random bird will be colonized with *Campylobacter* at the point of slaughter. Hartnett's risk assessment (2001) proposed a dynamic model for the spread of *Campylobacter* in a flock of chickens following the colonization of a single bird, and thus provides a realistic simulation of the complex event of in-farm colonization of birds. Following the rearing module, transport, processing, and consumption at home (including

cross-contamination) were also incorporated in the QMRA of Kelly et al. (2003). Based on an analysis of previously published data, the processing module predicted the effects of different steps (scalding, evisceration, chilling, etc.) on the level of *Campylobacter* on the chicken carcasses. Cross-contamination was modeled in the preparation-in-the-home module, based on previously published data as well. The QMRA presents an estimated risk of infection per person, per serving of chicken. In addition, the relative effects of various risk mitigation strategies in comparison with the baseline scenario were investigated. The simulation results concluded that among the strategies investigated, freezing all chicken products prior to consumption had the greatest effect on risk, followed by a reduction in the probability of cross-contamination during transport and in the home.

Bemrah et al. (2002) used the QMRA approach to assess the risk of human salmonellosis from the consumption of turkey cordon bleu in collective catering establishments in France. As described by Bemrah et al., turkey cordon bleu is made of reconstituted turkey, a slice of turkey ham, and a slice of processed cheese and coated with bread crumbs. In this study, the process was modeled and simulated using Monte Carlo techniques, from storage in the freezer of the catering establishment to the point of consumption. Data on *Salmonella* prevalence and levels on turkey cordon bleu and during preparation, cooking, and storage practices were collected specifically for this study from a number of catering establishments. Thermal inactivation kinetics of the isolated *Salmonella* strains were also determined and used in the cooking simulations. The QMRA identified that different cooking practices varied the impact on the estimated risk; oven cooking resulted in a risk of salmonellosis close to zero, whereas fryer cooking increased the risk due to the higher probability of achieving lower cooking temperatures. The effect of post-cooking storage on the overall risk of salmonellosis for both cooking scenarios was also investigated.

Alban, Olsen, Nielsen, Sorensen, and Jessen (2002) applied QMRA methodology to assess the risk of salmonellosis due to multidrug-resistant *Salmonella* Typhimurium DT104 from consumption of Danish dry-cured pork sausages. The aim of the study was to aid in deciding if pork contaminated with DT104 could be used for production of sausages without creating an increased public health risk. These questions were generated because of the higher prevalence of DT104 in imported pork meat versus Danish pork and also in consideration of Danish law, which indicated that meat with DT104 must be heat treated. Routine surveillance data, expert opinion, and data from pilot experiments were used to create inputs for the Monte Carlo simulations. However, due to the lack of data for DT104 specifically, surrogate data for *Salmonella* in general and *E. coli* were used for modeling production steps. The results of these simulations were transformed into values for DT104 considering the prevalence of DT104 in pork meat provided by Danish surveillance systems. The QMRA concluded that due to the low prevalence and low numbers of *Salmonella* in Danish as well as imported raw pork, and the 2- to 3-log reduction achieved

during processing, these meats may be used for the production of dry-cured sausages without representing an unacceptable health risk. However, the sensitivity analysis suggested that if pork meat with high prevalence of DT104 is used for the production of sausages, illness might occur.

Rosenquist, Nielsen, Sommer, Norrung, and Christensen (2003) developed a QMRA to assess the effect of different risk mitigation strategies on the number of cases of campylobacteriosis in Denmark associated with thermophilic *Campylobacter* spp. in chickens. This study was conducted in response to a recommendation by the Danish Veterinary and Food Administration as part of a strategy to control pathogens in foods. In the first module, the fate of *Campylobacter* spp. during slaughter and further processing of chicken carcasses into broilers was modeled using Danish surveillance data as well as previously published data. In a second module, preparation and consumption at home were modeled with respect to cross-contamination. Consumers' age and sex groups were incorporated as indicators of kitchen hygiene and the prevalence of safe or unsafe practices during meal preparation, utilizing previously published data. Monte Carlo simulations for different risk mitigation strategies concluded that the most significant reduction in risk was accomplished by a 2-log reduction of the level of *Campylobacter* spp. on the chicken carcass. It was also shown that eliminating cross-contamination from positive to negative flocks during slaughter had almost no effect on human incidence.

Nauta et al. (2005) proposed a standard poultry processing model using Monte Carlo simulation that may be used for any poultry-related QMRA, given the appropriate considerations. For example, if used on a QMRA for *Campylobacter*, no growth during processing may be assumed, as temperatures in poultry processing plants never reach the minimum growth temperature for this pathogen (30°C); if using the model for *Salmonella*, potential growth may be included. The proposed poultry processing model applies the same basic model in each processing step, considering bacterial inactivation, removal, and cross-contamination dynamics. This study discusses certain considerations with respect to the use of available microbiological data for risk assessment purposes. Nauta et al. (2005) questioned previous poultry-related risk assessments which considered a linear behavior to describe bacterial populations throughout processing. In such studies, poultry processing was modeled on the basis of the available microbiological data. Available microbiological data are generally presented as the mean of the change in concentration over a processing step; therefore, an additive process in the log scale was considered. However, linearity is unlikely when cross-contamination is dominant (Nauta et al. 2005). In an example for *Campylobacter* spp., this QMRA demonstrated that the effect of cross-contamination is dominant in carcasses with low initial levels of bacteria, and inactivation and removal are dominant in carcasses with high bacterial levels. As explained by Nauta et al., environmental contamination will contribute significantly to the carcass contamination only when the number of bacteria on the carcass is low. If it is high, the environmental contribution will be relatively low and negligible.

Perez-Rodriguez et al. (2006) demonstrated how QMRA can be applied to evaluate food processing/handling in relation to the achievement of food safety objectives (FSO) using Monte Carlo simulation. FSO specify the maximum frequency and/or concentration of a microbiological hazard that provides the appropriate level of protection to the public. In this risk assessment, different cross-contamination scenarios that may occur during handling and storage of cooked ham at the retail and consumer levels were modeled. Several cross-contamination scenarios were modeled with respect to pathogen transfer between product, gloves, and bare hands, providing a comprehensive simulation of cross-contamination dynamics. However, surrogate data were used to create transfer rate distributions due to the lack of specific data for *Listeria* spp. The potential growth of *L. monocytogenes* in home refrigerators was also considered. The output of the QMRA provided an estimate of the concentration of *L. monocytogenes* in cooked ham at the time of consumption, and thus it is possible to evaluate which processing/handling scenarios exceed an established FSO. The impact of different risk management interventions indicated that the highest risk corresponded to the use of the same gloves to handle contaminated meat and then sliced ham.

Uyttendaele et al. (2006) conducted a QMRA for *Campylobacter* spp. in poultry meat in response to European and Belgian regulations. The aim of the study was to analyze the relative impact of reducing the risk of campylobacteriosis associated with a decrease in the *Campylobacter* contamination level in raw chicken meat products. A meat preparation was defined as portioned or minced meat to which spices or other ingredients to improve sensory properties or texture might have also been added. The QMRA only considered a retail-to-consumption scope. Data for the prevalence of *Campylobacter* in poultry-based meat preparations were derived from the Belgian surveillance system. Surveillance data were provided as presence/absence of *Campylobacter* spp.; therefore, assumptions had to be made when constructing a distribution representing the level of contamination with *Campylobacter* spp. on poultry-based meat preparations. Consumer handling was modeled with respect to undercooking and cross-contamination. Cross-contamination was modeled by considering previously published data and assuming transfer rates between contaminated meat and a surface (for example, knife or cutting board). Undercooking prevalence was derived from published data. The QMRA presented three different approaches for describing the dose–response relationship, due to the limited number of studies on the human response to a dose of *Campylobacter* spp. Two of these approaches were based on the dose–response model presented in the preliminary QMRA for campylobacteriosis in broiler chickens developed by FAO/WHO (2002a), and the third one was based on the method described by Oscar (2004) in his QMRA for *Salmonella* and whole chickens. Based on the QMRA results, the authors suggested implementation of practices which would reduce the variability of the concentration of *Campylobacter* spp. in raw chicken meat products (which would correspond to a reduction in the prevalence of highly contaminated products).

National and International QMRAs

International agencies have driven significant initiatives aiming at the implementation of a risk analysis approach for the microbial safety of meat and poultry products. The Food and Agriculture Organization of the United Nations (FAO) in conjunction with the World Health Organization (WHO of the United Nations) have developed risk assessment programs that produced a number of meat-related QMRA examples (Table 23.2) as part of their microbiological risk assessment series. The series was initiated in response to the recognized social and economic burden created by foodborne illness worldwide. Also, as risk assessment may be used to justify more stringent standards for imported foods, its application can be relevant for trade purposes. Thus, the series aims at providing tools for understanding and, if possible, undertaking QMRA at the national level by developing examples of risk assessments considering a global or standard scenario to describe a food processing operation. Indeed, some of the "standard" processing parameters modeled may accurately describe the situation in many countries; however, it is acknowledged that regional differences may exist, for example, in prevalence data or consumption patterns as well as between the situation in developing countries and that of developed nations. Government bodies of different countries or regions (e.g., United States, New Zealand, European Union) have also developed comprehensive meat-related QMRAs for their individual situations. In this chapter, meat-related QMRAs developed by FAO/WHO will be discussed as a means to exemplify current global risk assessment approaches (Table 23.2). QMRAs developed by US agencies (i.e., FSIS) will also be discussed to demonstrate a national risk assessment approach (Table 23.3).

FAO/WHO presented a partially completed QMRA for *Campylobacter* spp. in broiler chickens as a result of expert consultations (FAO/WHO, 2001, 2002a), considering a farm-to-table approach and utilizing Monte Carlo techniques. Models for colonization and transmission of *Campylobacter* spp. in flocks, for cross-contamination during preparation in the home, and for thermal inactivation of *Campylobacter* spp. during oven roasting of whole carcasses were developed in this risk assessment. However, these models were not fully

Table 23.2 Quantitative microbial risk assessments for meat and processed meat of international scope

Pathogen	Meat/Poultry Product	Scope	Reference
Campylobacter	Broiler chickens	Farm to consumption	FAO/WHO, 2001, 2002a
Salmonella	Broiler chickens	End of slaughter process to consumption	FAO/WHO, 2002c
L. monocytogenes	RTE fermented meat products	Retail to consumption	FAO/WHO, 2002b

Table 23.3 Quantitative microbial risk assessments for meat and processed meat of national scope

Pathogen	Meat/Poultry Product	Scope	Reference
L. monocytogenes	RTE meat products	Retail to consumption	FDA/FSIS, 2003
L. monocytogenes	Deli meats	Production to retail	FSIS, 2003
L. monocytogenes	RTE meat products	Risk-based sampling of establishments	FSIS, 2007
E. coli O157:H7	Ground beef	Farm to consumption	FSIS, 2001
E. coli O157:H7	Tenderized and non-tenderized beef	End of processing to consumption	FSIS, 2002
Salmonella	RTE meat and poultry products	Risk associated with lethality standards	FSIS, 2005
C. perfringens	RTE and partially cooked meat and poultry products	Production to consumption	FSIS, 2005

implemented into the QMRA due to uncertainties attributed to the lack of important data and validation alternatives. Published and unpublished data were analyzed and used to generate a unified dose–response model for the probability of infection by *Campylobacter* spp. This data however carry a degree of uncertainty since it only included a limited number of strains, used a milk matrix, and considered only healthy volunteers in the feeding trial. Although no risk estimates or evaluation of risk mitigation strategies were provided at this stage of development of the QMRA, a comprehensive analysis of each step of the QMRA was provided, as well as detailed identification of data gaps.

A QMRA for *Salmonella* in broilers was also developed as part of the FAO/WHO microbiological risk assessment series (FAO/WHO, 2002c). A Monte Carlo simulation approach was taken, and events between the end of the slaughter process and consumption were modeled, including storage at retail, transport, and storage and cooking (or undercooking) at home. However, specific steps taken into consideration when modeling these processing and handling stages, especially mathematical relations, were not provided in the document. Cross-contamination during preparation at home was not included in the QMRA due to the lack of representative data and the complexity of this event. As part of the hazard characterization stage, a review of published dose–response models describing the relationship between an ingested dose of *Salmonella* and the probability of illness, as well as a review of outbreak data, was conducted. From this analysis, it was found that outbreak data could not be adequately described by available models, and a new dose–response model derived from the outbreak data was proposed. The results of the QMRA are

expressed as an estimate of the probability of illness in a year due to the ingestion of *Salmonella* on fresh whole broiler chicken carcasses with the skin intact and which are cooked in the domestic kitchen for immediate consumption. It was concluded from the overall QMRA simulations that a reduction in the prevalence of *Salmonella*-contaminated chicken was associated with a reduction in the risk of illness.

Also as part of the FAO/WHO microbiological risk assessment series, a QMRA for *L. monocytogenes* in ready-to-eat (RTE) foods was developed (FAO/WHO, 2002b). Four representative products of the RTE category were selected for this QMRA. Among these, fermented meat products, which include fermented dry and semi-dry sausages, were considered. Fermented sausages may be contaminated with *Listeria* spp. and are produced without any lethal processing step, but their final composition (lactic acid, salt, nitrite) prevents the growth of *Listeria* spp. during storage, thus representing a RTE product which does not support the growth of this pathogen. The scope of the QMRA includes events between the time of purchase and consumption (e.g., contamination at retail, growth before consumption, and consumption) using predictive models to describe the fate of *L. monocytogenes* in each step as a function of time and temperature. As opposed to the case of *Salmonella*, dose–response data from human volunteer studies with *L. monocytogenes* do not exist. As part of the risk characterization section of this QMRA, available dose–response models constructed using animal data as well as epidemiological data were thoroughly analyzed. However, none of these models were found suitable for the purposes of this QMRA. The QMRA constructed a set of simpler dose–response models based on the FDA/FSIS exposure assessment information (FDA-CFSAN & USDA-FSIS, 2001), simplified using an exponential dose–response relation, and differentiating between susceptible and healthy populations. The overall results of the QMRA are expressed as the number of illnesses in healthy and susceptible populations. Due to the moderate rates of consumption of fermented meat products and the high probability of inactivation of *L. monocytogenes* in the product during storage, the estimated risk per serving and number of annual illnesses were very low. The QMRA also answered specific questions with respect to the probability of illness associated with different ingested doses, with foods that support the growth of *L. monocytogenes* and foods that do not, and with populations of different susceptibility. It was concluded that, according to the models developed, nearly all cases of listeriosis result from the consumption of high numbers of the pathogen and that most of the cases of listeriosis are associated with the consumption of foods that do not meet currents standards for *L. monocytogenes* in foods. A reduction of risk for RTE foods that do not support the growth of *L. monocytogenes*, such as fermented sausages, could be achieved by reducing the frequency of products carrying high levels of contamination at retail.

A QMRA to assess the risk of severe illness associated with the consumption of RTE foods that may be contaminated with *L. monocytogenes* was developed

jointly by FDA and FSIS (2001). This QMRA was initiated as an evaluation tool to support the Healthy People 2010 government initiative aimed at reducing the incidence of foodborne listeriosis by 50% by the end of 2005. RTE foods with a documented history of contamination with *L. monocytogenes* were considered in this QMRA. Among RTE foods under evaluation, meat products such as frankfurters (heated and non heated), dry/semi-dry fermented sausages, deli meats, and pâté and meat spreads were included, representing products that support and do not support the growth of *L. monocytogenes*. Three population groups were considered in this QMRA, based on the FoodNet surveillance data: perinatal, elderly, and the remaining population. Apart from previously published data and expert opinion, a large amount of unpublished surveys acquired from state and federal public health offices as well as surveys conducted specifically for this QMRA were used to generate input data for Monte Carlo simulations. The scope of the QMRA covered events from retail to consumption, including changes in contamination levels during refrigerated storage and reheating in the home. The outputs of the QMRA were expressed as relative risk per serving and number of fatal infections per year in the United States, for each food category considered. The QMRA defines factors that may be used to calculate the relative risk of a particular RTE product versus another. Among the meat products considered in this risk assessment, the QMRA results indicated that in a per-serving basis, deli meats, non reheated frankfurters and pâté and meat spreads, were ranked in the high-risk category (>5 cases per billion servings). In a per-year basis, non reheated frankfurters were also categorized as high risk (>10–100 cases per annum), pâté and meat spreads fell in the moderate risk category (≥1–10 cases per annum), but deli meats were ranked as very high risk (>100 cases per annum). The high risk attributed to deli meats is a result of their high rates of contamination, their ability to support rapid growth of *L. monocytogenes* under refrigerated storage, the fact that they are often stored for extended periods, and are consumed extensively. Pâté and meat spreads may have high levels of contamination, but are consumed occasionally and in small quantities. For both per-serving and per-year basis, reheated frankfurters and dry/semi-dry fermented sausages were considered as low risk products (<1 case per annum; <1 case per billion servings), as a result of their ability to inhibit the growth of *L. monocytogenes*. Different scenarios and their effect on the QMRA illness estimates were evaluated. It was found that a 69% reduction in the risk of listeriosis could be obtained by assuring that all home refrigerators operate at 45°F or less. Reducing the storage time of deli meats, a food that can support the growth of *L. monocytogenes* to high levels during its normal shelf life, also resulted in a reduction in the number of illnesses, particularly in the elderly population. When evaluating changes in the contamination level of retail products such as deli meats, reducing contamination levels by itself would not have a major effect on reducing risk until reductions in contamination levels are large enough to affect the frequency of contamination.

The US FSIS developed a QMRA for *L. monocytogenes* in deli meats (Gallagher, Ebel, & Kause, 2003) in response to risk management questions regarding the effectiveness of food contact surface testing and sanitation regimes, as well as other interventions, in reducing the risk of contamination of *L. monocytogenes* in the finished product. Based on Monte Carlo techniques, a dynamic in-plant model was constructed to quantitatively correlate the presence of *Listeria* spp. in the plant environment with the presence of *L. monocytogenes* in deli meats at the retail level. In order to better address the risk management questions posed, this QMRA assumes that all *L. monocytogenes* in the RTE deli meat comes from food contact surfaces, and not from inadequate lethality treatment. The model assumes that a *Listeria* spp. reservoir (e.g., floor drains, AC ducts) exists in the plant and is capable of contaminating the food contact surface. After this initial contamination event, *Listeria* spp. on the food contact surface may be removed by sanitation, detected by testing, or remain on the surface and transfer to the food product. A ratio of *Listeria* spp. to *L. monocytogenes* was used to estimate the concentration of *L. monocytogenes* per gram of food. At this point, post-packaging interventions and finished product testing may reduce the concentration of *L. monocytogenes*, otherwise the food proceeds to retail. A detailed explanation of the mathematical basis used to create the model is provided in the document. The model was also presented on a user-friendly computer application, with graphic interfaces for data entry. The outputs of this QMRA (e.g., the concentration of *L. monocytogenes* in deli meats at retail) were used as inputs in the previously described retail-to-consumption FDA/FSIS *L. monocytogenes* QMRA, thus encompassing a production-to-consumption scope. The data generated show that a decline in the concentration of *L. monocytogenes* may be achieved by increasing surface testing and sanitation efforts. Regular food surface testing was shown to result in a reduction of the estimated median number of deaths among the elderly. The combination of post-processing and growth inhibitors was the only simulated scenario that led to a major decrease in the estimated total number of deaths. The sensitivity analysis concluded that retail concentrations of *L. monocytogenes* decrease as industry participation and intervention effectiveness increase.

In 2007, FSIS updated the 2003 *L. monocytogenes* risk assessment (Gallagher et al., 2003) in order to use it as a guide for the allocation of *L. monocytogenes* testing resources among FSIS-regulated establishments that produce post-lethality exposed RTE meat and poultry products. This is an interesting example of the evolution of a QMRA and an innovative approach for the risk-based allocation of resources. FSIS conducts a risk-based *L. monocytogenes* sampling program in establishments producing post-lethality exposed RTE meat and poultry products. The establishments scheduled for this risk-based sampling program are the ones with the greatest probability of producing RTE meat and poultry products contaminated with *L. monocytogenes*, as indicated by the risk assessment (USDA-FSIS, 2007). The 2003 *L. monocytogenes* QMRA (Gallagher et al., 2003) was modified, new data from the regulated establishments were incorporated, and

an algorithm that allocates sampling resources according to a risk ranking of the establishments was developed. Thus, establishments are ranked individually with respect to their likelihood of producing *L. monocytogenes*-contaminated product and resources are allocated based on that relative risk. The data sources used to calculate relative risk of each establishment and therefore its sampling frequency are the manner of compliance with the interim final rule to control *L. monocytogenes* (different combinations of post-lethality treatment, antimicrobial agents, and testing and sanitation measures), type of product processed, and volume of production and history of laboratory results for*L. monocytogenes*. For example, establishments with a history of negative *L. monocytogenes* samples which apply a post-lethality treatment and antimicrobial agents on the formulation to control *L. monocytogenes* may expect less frequent inspections than establishments with a history of positive *L. monocytogenes* samples and/or which only rely on antimicrobial agents to control *L. monocytogenes*. This risk-based approach to determine the frequency of sampling for *L. monocytogenes* in FSIS-inspected establishments has been successfully applied since 2005.

FSIS prepared a technical report describing a comparative risk assessment for tenderized and non-tenderized beef, considering the risk of illness posed by contamination with *E. coli* O157:H7 (USDA-FSIS, 2002). Under FSIS regulation, raw non-intact beef products (such as tenderized beef) contaminated with *E. coli* O157:H7 must be processed into ready-to-eat product, or they would be considered adulterated. Mechanical tenderization consists in repeated penetration of the muscle product (e.g., steak) by using needles or blades, in order to disrupt muscle fibers and tenderize the product. Steaks and roasts for hotel, restaurant, and institutional use in the United States are often tenderized. The process of tenderization is of concern because it may transfer dangerous pathogens from the surface of intact beef cuts to beneath the surface, thereby potentially protecting those pathogens from the lethal effects of cooking. Some of these pathogens may survive a cooking process that would kill all bacteria on the surface. Also, tenderization may result in cross-contamination of meat cuts that were originally not contaminated due to bacterial transfer by the tenderizing blades or needles. To determine if tenderization increases the risk of *E. coli* O157:H7 infection, a risk assessment was conducted to compare the estimated frequency of illness caused by tenderized and non-tenderized steaks. The model begins at the end of processing, with the degree of contamination on a produced steak. This level of contamination was determined with surrogate data for *E. coli* O157:H7 in ground beef, as estimated in a previously published risk assessment (USDA-FSIS, 2001). An adjustment factor was used to convert this data from pathogen concentration in ground beef to steaks. Potential growth during storage and handling was also modeled using the predictions of the risk assessment (USDA-FSIS, 2001). Data on different steak thicknesses and cooking practices (e.g., frying, grilling, broiling) from a previous study were used to model inactivation during cooking. Cooking temperature frequencies were extracted from the FDA Home Cooking Temperature Interactive Database for the category including beef, pork, and lamb.

Data from three *E. coli* O157:H7 human outbreaks were analyzed and modeled to generate a dose–response curve for the probability of illness due to consumption of the organism. The number of illnesses resulting from the consumption of cooked contaminated steaks was then calculated using this dose–response function. It should be noted that although this risk assessment is not based on Monte Carlo simulations, events are modeled using discrete frequency distributions. The final estimations of probability of illness by *E. coli* O157:H7 due to consumption of cooked tenderized and non-tenderized steaks were extremely low for both cases, although differences were observed between the estimates for tenderized (1 illness per 14.2 million servings) and non-tenderized product (1 illness per 15.9 million servings). This extremely low probability of illness was attributed in both cases to the unlikely survival of *E. coli* O157:H7 to typical cooking practices.

A draft report describing a detailed QMRA for the public health impact of *E. coli* O157:H7 in ground beef was developed by FSIS (USDA-FSIS, 2001). Although this is considered a draft, the work presented so far provides a large volume of comprehensive and valuable information on the risk of *E. coli* O157:H7 associated with ground beef. The scope of this QMRA considers a farm-to-fork approach, including on-farm events, slaughter, processing, and distribution steps, as well as cooking and consumption. Throughout the QMRA, estimates are based on Monte Carlo techniques. The QMRA models the prevalence and concentration of *E. coli* O157:H7 in live cattle, carcasses, beef trimmings, and, ultimately, a single serving of cooked ground beef. In the production module, the QMRA intends to quantitatively correlate the incoming status of the cattle with the outgoing status of the harvested meat, by using fecal *E. coli* O157:H7 prevalence data. For the purpose of this QMRA, imported beef is not considered different from domestic beef, but culled breeding cattle (from dairy and beef cow–calf herds) and feedlot cattle (steers and heifers sent to slaughter from feedlots) are separately modeled in this risk assessment. Seasonal variation in the prevalence of *E. coli* O157:H7 in the herd was incorporated in the production module; there appears to be a high prevalence season (June to September) and a low prevalence season (October to May). Published data suggest that *E. coli* O157:H7 prevalence does not change during transport from farm to slaughter plant; therefore, no effect from transport is included in this model. The production model outputs are distributions for cattle prevalence just prior to slaughter. These outputs become the inputs for the slaughter module to follow. The production module results predict that feedlot cattle are more likely than breeding cattle to be infected. Also, regardless of cattle type, higher frequencies of infected cattle enter slaughter plants during the June to September period than during October to May. In the slaughter module, the slaughter of breeding cattle and feedlot cattle during high and low prevalence seasons is also considered separately. The slaughter module includes seven steps: arrival of live cattle at slaughter plant, dehiding, decontamination, evisceration, final washing, chilling, and carcass fabrication (i.e., creation of trim). The slaughter module outputs are distributions of *E. coli* O157:H7 in

combo bins (containers that hold 2,000 pounds of meat trim) and trim boxes (60 pounds). Although only a fraction of infected cattle results in contaminated carcasses, thousands of pounds of meat trim from these carcasses are combined in the grinding process. Consequently, although the number of *E. coli* O157:H7 organisms in these grinder loads may be quite low, the proportion of grinder loads that contain at least one *E. coli* O157:H7 organism is expected to be high. In the preparation module, the effects of handling and cooking on the amount of *E. coli* O157:H7 in a serving of ground beef are modeled. The consumption of ground beef as hamburger patties and as a formed major ingredient (e.g., meatballs, meat loaf) is considered. Although cross-contamination could be a potential source of *E. coli* O157:H7 in ground beef products, it was not considered in the scope of this QMRA. The preparation module includes six steps: grinding, storage during processing by the retailer or distributor, transportation home or to an institutional environment (hotel, restaurant, etc.), storage at the home or institution, cooking, and consumption. The amount of ground beef consumed was considered to vary depending upon the age of the consumer and the location where the meal was consumed. Predictive models were used to estimate the growth of *E. coli* O157:H7 as a function of temperature during storage and transportation. In the case of frozen storage, a decline in *E. coli* O157:H7 levels, according to published data, was considered. Data from national surveys were used to model storage and cooking temperatures as well as consumption patterns by age and location. The preparation module output consists of a single exposure distribution for the frequency and extent of *E. coli* O157:H7 contamination consumed in a year. Detailed considerations for each module as well as input data sources are provided on the report. Due to the lack of data on the dose–response relationship for *E. coli* O157:H7 and human illness, a dose–response curve developed for this QMRA was derived from an estimation of the number of *E. coli* O157:H7-related illnesses attributable to the consumption of contaminated ground beef and an estimation of the likelihood and level of *E. coli* O157:H7 in cooked ground beef servings. Data from clinical studies with surrogate microorganisms were used to generate upper and lower bounds of the proposed dose–response curve. The proposed dose–response curve was validated against outbreak data. In the risk characterization section, risk estimates were provided for "typical" individuals, a community in a simulated outbreak scenario, and the US population, considering differences in seasonal exposure, host susceptibility, and location of meal consumption. The median probability of illness for the general US population due to *E. coli* O157:H7 from a serving of ground beef was estimated to be about 1 in every 1 million servings. A sensitivity analysis was also provided in this draft report, where factors that significantly influence the occurrence and extent of *E. coli* O157:H7 in ground beef were identified. Among others, factors such as the size of the *E. coli* O157:H7-contaminated carcass surface area, decontamination steps during slaughter, carcass chilling, number of *E. coli* O157:H7 in the grinder loads, and refrigerated/frozen storage of ground beef were found to influence the extent of *E. coli* O157:H7 contamination in ground beef.

In order to determine a correlation between the required lethality of a process and its associated public health risk, a risk assessment of the impact of lethality standards on salmonellosis from RTE meat and poultry products was developed (Decisionalysis Risk Consultants & USDA-FSIS, 2005). The lethality of a process in this context refers to the achievement of a specific log reduction of *Salmonella* spp. that may be present in raw materials. This risk assessment considers only the risk posed by salmonellae that contaminate raw materials and may survive the lethality step during processing. The RTE meat and poultry products category was divided taking into account whether the product was cooked, cured, fermented, or dried; representative products were then chosen for each category. The processing and handling steps modeled in this risk assessment were *Salmonella* contamination in raw materials, surviving organisms in the finished product after the lethality treatment, compliance with the required level of lethality, growth during storage, decrease in pathogen concentration during reheating by the consumer, and probability of illness from the surviving pathogens. In this risk assessment, a probability (rather than a distribution) was assigned to each step and the model estimates were not constructed based on Monte Carlo techniques. Limitations and uncertainties included the uncertainty associated with the extent to which consumers reheat different RTE meat and poultry products, pathogen growth during storage, and probability of illness due to consumption of very small numbers of pathogens. Risk estimates were provided for a variety of process lethality scenarios: a 5-log reduction for all product categories, a 6.5-log reduction for cooked products (a 7-log reduction if they contain poultry), and a 5-log reduction for all other products or a 6.5-log reduction for all product categories (again, a 7-log reduction if they contain poultry). The impact of these simulated lethality standards was estimated based on the calculated post-processing pathogen burden as the difference between the pathogen load on the raw materials and the lethality standard. Careful considerations were taken in order to estimate the pathogen load on the raw materials. As factors that can influence the actual lethality achieved, compliance and thermal process safety factors were incorporated into the model. When the lethality of the process requires achieving a specific log reduction in the coolest point of the product, most of the mass of the product will experience a higher level of lethality than the coolest point. Due to quality considerations, some products are more likely to achieve higher process lethality than others. These issues are taken into account as "thermal process safety factors". A predictive growth model was used to estimate growth during storage as a function of temperature. Although pH and/or water activity characteristic of several of these RTE meat and poultry products may have an effect on the growth of *Salmonella*, only temperature was considered in this risk assessment. Storage temperature data were obtained from surveys and assumptions were made with respect to storage time. The latter were compatible with those assumptions considered on the FDA/FSIS QMRA for *L. monocytogenes* on RTE foods (FDA-CFSAN & USDA-FSIS, 2001), and adjustments were made for products that either do or do not

allow for the growth of the pathogen. Reheating of the product was categorized as never, rarely, usually, or always and different log reductions were attributed to each category. The dose–response curve presented in the FAO/WHO (FAO/WHO, 2002c) QMRA for *Salmonella* in eggs and broilers was incorporated into this risk assessment. Risk estimates were provided by considering the estimate of risk per million kilograms and the level of consumption associated with the different product categories. When all product categories were considered in the 5-log reduction lethality simulated scenario, cooked chicken (nuggets, tenders, non-deli) was by far the largest contributor to the estimate of number of cases of salmonellosis per year. For the simulated scenario of 6.5-log reduction for cooked products (or 7-log if they contain poultry) and a 5-log reduction for all other products, cooked chicken (nuggets, tenders, non-deli) was still the greatest contributor, but to a much lesser extent. Under the latter scenario, other products that were found to contribute significantly to the estimated risk were those under the category "fermented or direct acidified, uncooked, shelf stable" (e.g., salami, uncooked pepperoni) and "dried, including heat treatment" (e.g., beef jerky). A sensitivity analysis for the different events modeled in this risk assessment was presented and included variations of the assumptions considered for each step, such as prevalence data, level of compliance, thermal process safety factor, reheating, growth rate, and consumption volume.

A risk assessment for *Clostridium perfringens* in RTE and partially cooked meat and poultry products was prepared by FSIS (Crouch & Golden, 2005). Two main objectives were targeted in this QMRA: (1) to evaluate the public health impact of changing the allowed maximal growth of *C. perfringens* during cooling after the cooking step (referred to as "stabilization") of RTE and partially cooked meat and poultry products and (2) to examine whether the steps taken to limit the growth of *C. perfringens* in these products would also be adequate to protect against the growth of *C. botulinum*. Under FSIS regulation, performance standards for the production of all RTE and partially cooked meat and poultry products propose limiting the multiplication of *C. perfringens* to a maximum of 1 log within the product during manufacture. In particular, this QMRA was designed to estimate the probability of illness if the allowed growth of *C. perfringens* is raised to 2 logs or 3 logs, and to estimate the relative growth of *C. botulinum* associated with the increased growth allowance for *C. perfringens*. The scope of the QMRA considers a processing plant-to-table approach. Based on Monte Carlo simulations, *C. perfringens* spore and vegetative cell contamination on raw meat and poultry products (and spices, if part of the formulation) was simulated from the production plant to the point of consumption. The processing and handling steps modeled included original contamination of raw meat and poultry with *C. perfringens* spores and vegetative cells, contamination after manufacture, during storage between manufacture and retail, during storage between retail and consumption, and during preparation in the home (reheating and hot-holding). The model considers that cooking will inactivate vegetative cells, but will stimulate spore germination and partial cooking may permit survival of a fraction of the original vegetative cells.

The model also considers the fact that germinated spores and surviving vegetative cells will grow until the food is cooled to a temperature that prevents such growth. Subsequent processing, storage, transport, and preparation steps each change the concentration of vegetative cells to some extent, mainly due to temperature variations. The foods included in this QMRA were identified from meat-containing food servings from the Continuing Survey of Food Intakes by Individuals (CSFII). The selected foods were separated into four categories based on their likelihood to be reheated and/or hot-held before consumption. For RTE foods, the concentration of *C. perfringens* spores and vegetative cells was calculated as a result of heat treatment, while for partially cooked products, these concentrations were assumed to be the same as in raw meat products. In both cases, previously published studies were used as data sources. Data for the prevalence and levels of contamination with *C. perfringens* spores in spices were also extracted from previous studies. The model also considers that only a fraction of the *C. perfringens* in given product actually represents type A, CPE-positive cells (i.e., those that produce *C. perfringens* enterotoxin and are able to cause diarrheal disease), and a conversion derived from published data was applied to estimate the fraction of type A *C. perfringens* cells. Predictive growth models were used to estimate the germination of spores and growth of vegetative cells for both *C. perfringens* and *C. botulinum*. Growth during stabilization, chilling, and secondary cooking steps were all included as variables in the model. A practical approach was taken and fixed values of growth (0.5–3.5 logs) for all RTE and partially cooked foods were assigned using point distributions. Inactivation of *C. perfringens* vegetative cells at temperatures below 10°C was considered when modeling storage and transport, as derived from published data. Spontaneous germination of spores during storage and transport as well as germination during reheating was included in the model. The temperature and duration of storage, reheating, and hot-holding were modeled from survey data. A dose–response curve for the probability of illness (diarrhea) associated with the ingestion of *C. perfringens* cells was constructed from human feeding data presented in previous studies. An average dose–response curve was obtained from data on different strains. Finally, the number of vegetative cells consumed in a serving and the probability of those cells causing illness is calculated for each serving. Considering the total number of servings of RTE and partially cooked foods in the United States per year, the risk of diarrhea increased from 74,000 to 149,000 per year for the simulated 0.5 logs and 3.5 logs of growth during stabilization, respectively. Surprisingly, the QMRA also indicated that retail and consumer storage temperature had a significant effect on the estimated risk. For storage temperatures below the minimum for growth of *C. perfringens*, illness was very unlikely. Illness became much more likely for storage temperatures above this minimum, and the length of storage was found to usually be sufficient enough to allow growth to stationary phase. The QMRA also concluded that it is not possible to make any definitive claims regarding limits to *C. botulinum* growth with reference only to *C. perfringens* growth, mainly due to differences in the growth

characteristics of these microorganisms. Any measures taken to reduce or prevent growth of *C. perfringens* will not necessarily do the same for the growth of *C. botulinum*. An interesting analysis of different scenarios on the estimated risk of illness included the potential outgrowth of *C. perfringens* by spoilage organisms and the effect of consumer detection of high concentrations of *C. perfringens* (spoiled product). A sensitivity analysis for the model parameters was also included in the QMRA.

Conclusions

Quantitative microbiological risk assessments for different pathogen/meat or poultry product combinations conducted independently at the national level and at the international level can provide valuable information for different food safety issues relevant to the meat and poultry industry. Among the studies discussed in this chapter, examples were given that demonstrate the successful application of this tool to aid in the analysis of a wide variety of food safety issues: the achievement of food safety objectives, the risk associated with different lethality standards, the establishment of microbiological criteria, and the effect of risk mitigation strategies, among others. Thus, apart from providing estimations of the overall probability of illness associated with a food product, QMRA has the ability to organize relevant data and identify food processing/handling steps that significantly impact the overall risk associated with the consumption of a particular food product.

The quality of the data sources used to develop a QMRA is of utmost importance as it will determine the level of accuracy of the risk estimations produced. How closely the QMRA model describes the actual scenario being studied will also significantly affect the accuracy of its conclusions. Validation of QMRA risk estimates may be undertaken with respect to epidemiological data; however, the generality of the majority of epidemiological data available makes this a challenging task, especially for QMRAs considering specific food products within a larger food product category or when specific food handling conditions are being evaluated.

Although independent, national and international QMRAs can indeed provide useful information; important differences among these studies are evident. For example, several QMRAs developed by international agencies consider a general or standardized estimation of risk for common pathogen/product combinations as a means to exemplify the application of this tool. International risk assessments often incorporate data from different countries, and thus its results should be carefully interpreted. These studies have worldwide impact and have been developed by multidisciplinary teams of renowned experts. The information provided has proven to be extremely valuable for the application of QMRA to more specific and locally relevant food safety scenarios. National level QMRAs developed by government agencies provide a more accurate

estimation of risk for the local situation, especially considering the availability of large amounts of relevant data that can be incorporated into these studies, as well as the inclusion of specific regional differences (e.g., pathogen prevalence in the raw materials or food handling practices). Independent QMRAs have been undertaken with a national scope, but specific issues can also be thoroughly examined without necessarily providing an estimated overall number of illnesses. Among such studies, the prevalence of pathogens at the farm level only (the outputs of which may be used as inputs in further processing and consumption QMRA stages), the achievement of microbiological criteria, or the evaluation of particular risk mitigation strategies have been the focus of a number of QMRAs.

Common to all these studies, despite taking an independent, national, or international approach, is the identification of significant data gaps that increase the uncertainty of the QMRA results. Significantly, data on the dynamics and mechanisms of cross-contamination are limited and thus several QMRAs have opted for not including this event on their model. However, it is acknowledged that cross-contamination may have a significant effect on the risk estimates provided. Also, the dose–response relationship for a number of pathogens is currently unknown; in many cases this has been overcome by the systematic analysis of available data from surrogate microorganisms, animal studies, and/or outbreak data to generate estimated dose–response curves.

Finally, although different QMRAs have been developed for different pathogen/product combinations, as well as for different purposes and scopes, portions of the information provided may be incorporated in other QMRAs. For example, dose–response curves for a specific pathogen should be applicable to estimate the risk associated with its consumption in different food products. On the other hand, data on handling practices such as storage time and temperature of a particular food product, often derived from surveys, should be useful to estimate the risk associated with the consumption of different pathogens in this particular food product.

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Chapter 24
Quantitative Risk Assessment of Bovine Spongiform Encephalopathy

Toshiyuki Tsutsui and Fumiko Kasuga

Introduction

Bovine spongiform encephalopathy (BSE) is a progressive neurological disease of cattle affecting the central nervous system and was first diagnosed in the United Kingdom (UK) in 1986 (Wells et al., 1987). This disease is one of the transmissible spongiform encephalopathy (TSE) which includes Creutzfeldt–Jakob disease (CJD) in humans and scrapie in sheep. The causative agent of TSE is considered to be an abnormal form of prion protein. However, the details of its pathogenic mechanism have not been fully identified. Scrapie, which causes neurological symptoms in sheep and goats, has existed in the UK for 200 years (Hoinville, 1996) and spread across the rest of the world in the 1900s (Detwiler & Baylis, 2003). There has been no report so far that scrapie can be transmitted to humans. Initially, BSE was also considered as a disease affecting only animals. However, a variant type of Creutzfeldt–Jakob disease (vCJD) was first reported in the UK, and exposure to a BSE agent was suspected (Collinge, Sidle, Meads, Ironside, & Hill, 1996). vCJD is clinically and pathologically different from the sporadic type of CJD, and age at clinical onset of vCJD is younger than sporadic type (Will et al., 1996). Since the UK government announced the possible association between BSE and vCJD in 1996, BSE has become a huge public health concern all over the world. Of particular concern about vCJD, the fatal disease in younger age, distorted consumer confidence in beef safety, and as a result reduced beef consumption has been seen in many BSE-affected countries.

The rise in public concern has promoted attitudes for using science-based measures for food safety assurance. As a science-based approach, risk assessment has become recognized as an efficient tool in the last two decades, particularly after the WTO-SPS agreement stated risk assessment as a tool for decision-making on border control measures in 1995. Risk assessment of

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chemical residues in food has a long history of application, and methodology has been well studied. However, the assessment of food risk caused by microbial organisms has rarely been performed. In general, microbial risk assessment is affected by various uncertain factors, such as growth or deactivation of the pathogen and the dose–response relationship between pathogen and host with various susceptibilities. In addition, available data are often limited to enable proper risk assessment. Generally, there are many constraints to conducting risk assessment on the diseases such as BSE because of the shortage of data and knowledge on the disease itself. However, since various measures against BSE are now required from a public and animal health point of view, the risk assessment has been widely applied as an important part of the decision-making process.

Risk Assessment of BSE

Approaches used for the risk assessment can be classified into two types: qualitative and quantitative. Qualitative approach expresses the magnitude of risk in a descriptive way, such as high, low, negligible, without numerical calculation of probability or likelihood. This approach is appropriate to evaluate risk comprehensively from a broad range of aspects. It is also used when available data or time are limited. On the other hand, quantitative approach quantifies all issues influencing the risk and provides quantitative outputs, such as likelihood or incidence. Outputs from the quantitative risk assessment are often favorable because they are easy to interpret and can compare different risk mitigation measures directly.

To conduct the quantitative risk assessment on BSE, certain assumptions are unavoidable because BSE still has many unknown features. For example, when the human health risk of BSE is considered, it is favorable to express the outputs as the number of disease cases in humans. However, since vCJD is the fatal disease, experimental challenges are hardly performed to acquire infectious doses of the BSE agent for humans. Even for cattle, the long incubation period makes it difficult to collect direct experimental evidence for estimating the dose–response relationship from inoculation studies. These experiments require appropriate facilities and large cost to maintain inoculated cattle for a long period of time. In the present circumstance, the cattle inoculation data mostly rely on previous studies conducted in the UK. In addition, the correction of epidemiological evidence in the field is often difficult, because the infection occurred long ago and the incidence of BSE in the population is very low. The lack of diagnostic methods in live cattle is another constraint because it makes it difficult to estimate disease prevalence in the standing cattle population. Even though these constraints exist, various quantitative risk assessments have been conducted on BSE. Most of the risk assessment models control uncertainty by using mathematical or stochastic methods.

Quantitative Approaches for the Food Safety Risk Assessment of BSE

Pathway Modeling Approach

An approach often used in quantitative assessment is a pathway analysis. This type of analysis follows the risk pathways according to possible events affecting the magnitude of risk. The event tree, which represents plausible pathway scenarios toward the occurrence of adverse effect or defined end points, is often established first. Then, the likelihood of occurrences in each scenario is determined taking account of the uncertainty and variability of parameters incorporated. This type of approach is frequently used in exposure assessment of quantitative microbiological risk assessment on food safety. For BSE, this approach was used for the risk assessment of sewage sludge (Gale & Stanfield, 2001), milk replacer (Paisley & Hostrup-Pedersen, 2004), disposed cattle (Comer & Huntly, 2003), and meat bone meal (Yamamoto et al., 2006). In the food safety area, DNV (1997) and Cooper and Bird (2002) assessed the human exposure risk of BSE infectivity through beef consumption in the UK by building event tree-type models. Usually these models build in stochastic effects for reflecting potential uncertainty and variability by the Monte Carlo method. This approach is easy to follow for non-experts and can identify data required for further refining the models. One disadvantage of this method is the difficulty of verification of the model. The appropriateness of the model is ensured by validating the model from the plausible outputs and reasonable behavior of the model. Furthermore, Gravenor and Kao (2003) mention that setting various pathways may dissipate the risk of each pathway in the end.

Epidemiological Modeling Approach

Another type of approach used so far in the food safety risk assessment of BSE is an epidemiology-based approach. This approach predicts the human exposure to BSE agents in the population on the basis of the observed incidence of BSE or vCJD. Ferguson and Donnelly (2003) and Arnold and Wilesmith (2003) used mathematical models to predict the number of infected animals entering the human food chain under different scenarios of risk mitigation measures. They used back-calculation methods for predicting the future course of the epidemic of BSE (Anderson et al., 1996; Ferguson, Donnelly, Woolhouse & Anderson, 1997) and vCJD (Ghani, Ferguson, Donnelly, Hagenaars, & Anderson, 1998). This method reverse estimates the number of infections from the number of observed cases, evaluating the factors such as incubation period and time-dependent exposure. Then, future cases are estimated forward from the estimated infections. This method provides an insight into the epidemic course in the population with reasonable validation techniques. Another advantage of this approach is that the

detailed dose–response relationship between agent and host species is not required for model building. However, this mathematical approach is sometimes difficult to understand for non-experts, and output values tend to be referred to without considering underlying assumptions. In addition, it is stated that this approach provides little information about recent infections due to the long incubation period of BSE and was not appropriate for long-term prediction (Supervie & Costagliola, 2007).

Examples

Here, we explain the issues related to conducting quantitative risk assessment on BSE by discussing two examples. First is a stochastic modeling approach to evaluate the effectiveness of BSE screening tests at the slaughterhouse. Among the control measures taken against BSE, cattle testing schemes at slaughter for detecting BSE-infected cattle are varied among countries. Most European countries test cattle over 30 months of age at slaughterhouses, while some other European countries test over 24 months of age. Japan has targeted all cattle irrespective of their age. The first model was built to compare the efficacy of testing schemes under various targeting age scenarios (Tsutsui & Kasuga, 2006). The other model used an epidemiology-based modeling approach. This estimates the number of infected cattle entering the food chain using the observed BSE incidences (Yamamoto, Tsutsui, Nishiguchi, & Kobayashi, 2008). This approach uses a simulation method, rather than mathematical equations, for the back-calculation.

Stochastic Modeling Approach

Model Structure

When the pathway model is considered for assessing the human health risk caused by the BSE agent, an event tree may need to follow a risk pathway from the BSE-infected cattle to the vCJD patients (Fig. 24.1). Ideally, the number of vCJD occurrences should be estimated from the number of BSE-infected cattle in the total cattle population in order to obtain the absolute risk to the human population. However, the number of BSE cases (Part 1) and the vCJD cases (Part 3) can be estimated only under certain assumptions with considerable uncertain input parameters. It is of concern that modeling all events through this pathway results in amplifying uncertainty in the model outputs. Since the purpose is to evaluate the impact of alternative testing schemes on the risk to human health, Part 2 provides enough information to see those effects although the difference of absolute risk is not acquired. In quantitative risk assessments, modeling is often focused on only the key process of the risk pathway to compare different scenarios. This could remove unnecessary uncertainty from

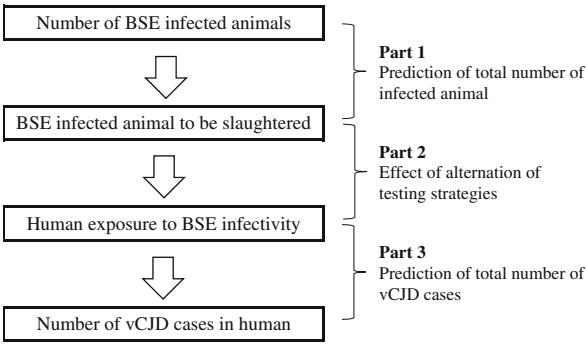

Fig. 24.1 Ideal pathway for modeling

the risk assessment models, but still contributes to decision-making. In Part 2, the purpose of modeling is specified more concretely by comparing the efficacy of screening tests when a BSE-infected animal is slaughtered under different testing schemes.

The model structure is outlined in Fig. 24.2. First, the model estimates infectivity accumulated in the brain stem of infected cattle at the time of slaughter. Then, the estimated infectivity is examined and determines whether the titer is higher than the detection limit of the screening test. The infected cattle with negative results are expected to enter the food chain, while the whole carcass of positive animals is removed. The different age targeting test schemes are incorporated into the model to compare their impact on outputs.

The unit of the risk measurement is another important item to be carefully selected in the risk assessment model. This is profoundly associated with structures and outputs of the model. The choice of unit should be made considering the end point of risk assessment and the ease of treating it in the model. Since the aim of modeling here is to assess the relative change of the exposure by different scenarios, murine intracerebral ID_{50} (m.i.c. ID_{50}) units can be used as the unit of measure for infectivity. This avoids related uncertainty on the

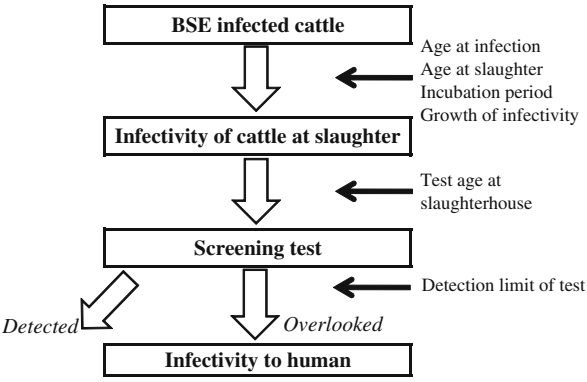

Fig. 24.2 Model structure

conversions from mouse units to cattle/human units and enables use of mouse bioassay data directly. Stochastic effects can be generated by Monte Carlo simulation.

Modeling Infectivity of Cattle at Slaughter

The central part of this risk assessment model is to model the infectivity of cattle at slaughter. To estimate the infectivity at slaughter, we need to know when the infected cattle are slaughtered during the incubation period and how much infectivity is accumulated at that stage. We could assume that each infected individual has a unique incubation period, *Inc_period*, derived from the assumed distribution of incubation periods. If this animal is infected at age of *Inf_age*, the potential age of the disease development, *D_age*, is described by adding *Inc_period* to *Inf_age*.

$$D_age = Inf_age + Inc_period$$

The underlying assumption here is that incubation period is not affected by the age at infection. Age of slaughter, *S_age*, is estimated from the age distribution of slaughtered cattle derived from the national data. Since we know the time of clinical onset, *D_age*, the time period until clinical onset at slaughter, *C_period*, can be calculated by subtracting *S_age* from *D_age*.

$$C_period = D_age - S_age$$

If D_*age* < S_*age*, we could assume that an infected animal develops the disease on a farm and does not come to a slaughterhouse. The process of modeling until this point is described in Fig. 24.3.

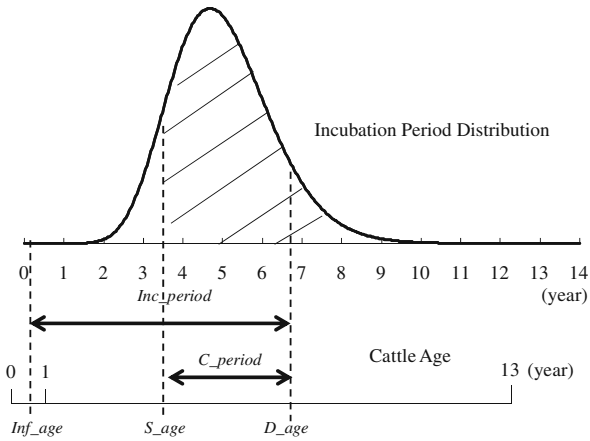

Fig. 24.3 Incubation period distribution and modeling approach for time left at slaughtering before estimated clinical development

The infectivity titer of the spinal cord at clinical stage, *C_titer*, was measured by mouse bioassay. If we know the way that the infectivity titer increases during the incubation period, the infectivity titer at slaughter, *S_titer*, can be estimated from *C_period*, the time period until clinical onset at slaughter, and *C_titer*. It could be assumed that the infectivity in cattle increased during the incubation period with infectivity titer doubling in a certain period, *Do_period*. The model calculates infectivity at the time of slaughter backward from the infectivity of *C_titer* by the time period of *C_period*, assuming that cattle develop clinical signs when the infectivity reaches a certain level. This also assumes that BSE infectivity increases in a similar way before clinical development irrespective of the duration of incubation period. The equation for calculating infectivity at the time of slaughter, *S_titer*, is as follows:

$$S_titer = C_titer/2^{(C_period/Do_period)}$$

Then, if *S_titer* is higher than the estimated detection limits of the applied test, the infected animal is detected. If it is lower, the infected cattle are overlooked and enter the food chain.

Input Parameters

Age at Infection

Epidemiological studies in the UK have suggested an age-dependent risk of BSE infection, and younger animals have a higher risk of infection (Arnold & Wilesmith, 2003; Ferguson et al., 1997; Anderson et al., 1996; Wilesmith, Ryan, Hueston, & Hoinville, 1992). Supervie and Costagliola (2007) and Calavas, Supervie, Morignat, Costagliola, and Ducrot (2007) considered that the BSE infection in France mostly occurred in cattle between 6 and 12 months of age by using mathematical modeling studies. The first year of life is presumed to be a high-risk period for BSE infection in the international guidelines (OIE, 2007). Age at infection could vary among countries due to the difference in cattle management systems. We assumed even probability of infection during the first year of life according to OIE guidelines.

Incubation Period

Anderson et al. (1996) derived a gamma distribution for the BSE incubation period from the UK epidemic. Later, Ferguson et al. (1997) reported that a mechanistic distribution was better fitted using a similar approach. Calavas et al. (2007) fitted a gamma distribution with slightly different parameters to the French BSE epidemic. These distributions are based on the analysis of the epidemiological data on the BSE epidemic in specific countries, mostly in the UK. However, the number of BSE cases observed in the UK is substantially higher than those in other countries, such as Japan, and thus the exposure of

cattle to BSE infectivity differs. Lower exposure dose is known to prolong the incubation period in a cattle bioassay study (Wells et al., 2007). For countries with a small number of BSE cases, it is impossible to estimate the incubation period from the original data. Therefore, we used a gamma distribution derived from the UK epidemic for the assumed incubation distribution. Then, the influence of lower exposure dose was tested by shifting the distribution toward a longer time period in the sensitivity analysis. Because of the large uncertainty regarding incubation period in the field, the influence of incubation period needs to be examined by sensitivity analysis with alternative distributions in any case.

Age at Slaughter

Age at slaughter depends on the demography and cattle management system in situations considered. In countries where beef industries are dominant, age at slaughter is generally younger than the intensive dairy countries. In Japan, the detailed data are obtained from the database of cattle identification system as detailed in Fig. 24.4.

Infectivity at Clinical Stage

Clinically affected cattle are considered to have infectivity of between 10^3 and 10^5 m.i.c. ID_{50}/g in the central nervous system (CNS) (SSC, 2002). In the bovine unit, Comer and Huntly (2003) proposed to use a log-normal distribution with a mean of 90 bovine oral ID_{50}/g of CNS tissues for risk assessment study, while Cooper and Bird (2002) used 10 b.o. ID50/g in their risk assessment. Attack rate studies using cattle indicated one cattle oral ID_{50} was equal to $10^{2.8}$ m.i.c./i.p.

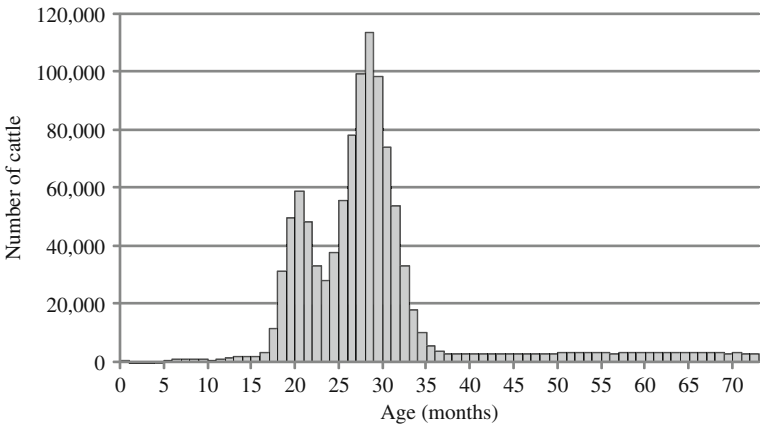

Fig. 24.4 Age distribution of slaughtered cattle in Japan

ID_{50}/g (95% CI: $10^{2.1}$–$10^{3.5}$) (Wells et al., 2007). For use of the unit of infectivity, route of inoculation, animal species, and breed of host need to be considered carefully. We assumed an infectivity of $10^{4.7}$ m.i.c. ID_{50} (median value of 10^3 and 10^5 m.i.c. ID_{50}) at clinical onset.

Doubling Time

Many models assumed that the infectivity in cattle increased during the incubation period, with infectivity titer doubling every 1.5 or 2 months (Comer & Huntly, 2003; de Koeijer et al., 2004; Ferguson & Donnelly, 2003). The exponential growth of infectivity, which is described in terms of the time required to double infectivity (doubling time), was derived from a scrapie pathogenesis study in hamsters (Beekes, Baldauf, & Diringer, 1996).

As an example, infectivity growth curves with a doubling time of 1, 2, or 4 months with a 60-month incubation period are shown in Fig. 24.5. Shorter doubling time, which means rapid increase of infectivity, results in a steeper growth curve of infectivity in the final stage of the incubation period as detailed in Fig. 24.5. These assume steep rises of infectivity at the final stage. In the model that we described, the rapid increase of infectivity causes the rapid decrease of infectivity toward the beginning of the incubation period, since the level of infectivity at the end of the incubation period is defined in advance. We assumed that the doubling time is somewhere between 1 and 2 months for each individual animal and applied a uniform distribution between the two values in the model. This parameter must be tested by the sensitivity analysis.

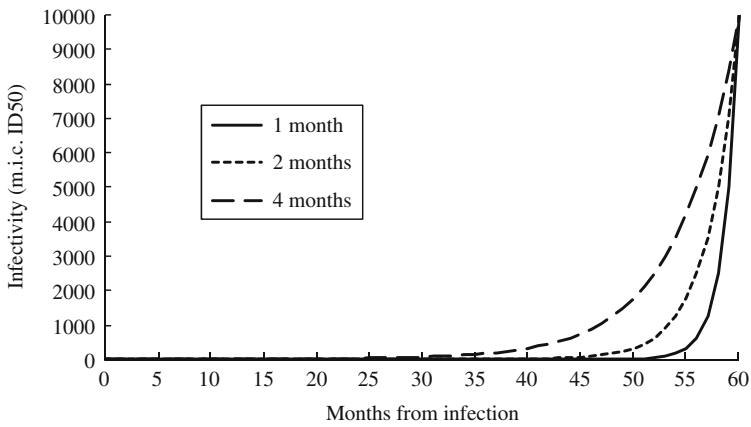

Fig. 24.5 Growth of the BSE infectivity during incubation period with a doubling time of 1, 2, or 4 months

Detection Limit of Infectivity by Test

In the evaluation of screening tests (SSC, 1999), 20 of 20 samples diluted 10^2-fold from the original brain samples of BSE-infected cattle with infectivity of $10^{3.1}$ m.i.c. ID_{50}/g were positive with ELISA, and 18 of 20 samples diluted $10^{2.5}$-fold were positive using the same ELISA kit. Detection limits can work on the all-or-nothing basis to determine individual animal test results, as we used $10^{3.1}$ m.i.c. ID50/g for the threshold value. Alternatively, chance effect can be built into the model using a binomial probability.

Outputs

Here, we described results of the comparison of the impact of different testing scenarios on BSE infectivity destined for the human food chain in Japan. The details of parameters used are described in a published article (Tsutsui & Kasuga, 2006). The maximum expected fraction of BSE-infected cattle that would be detected by screening tests occurs when all slaughtered cattle are tested (Table 24.1). But even taking this scheme, the fraction was only 20%. Testing only cattle aged over 20 or 24 months retains more than 96% efficacy when compared with all cattle testing scheme, while testing only cattle beyond 30 months would retain 77%. It is considered that those overlooked animals below the age limits have relatively low infectivity, because these cattle are mostly in the early stages of the incubation period. Infectivity entering the human food chain is further reduced by the removal of risk materials such as brain and spinal cord at slaughterhouses, as our original study indicated (Tsutsui & Kasuga, 2006).

The result of sensitivity analysis is shown in Fig. 24.6. Among the input parameters, the time required for doubling BSE infectivity has a significant impact on the probability of detecting BSE-infected cattle by screening tests. The probability of detection becomes considerably higher for all testing strategies, except testing those cattle beyond 30 month of age, when doubling time was assumed to be longer than 1 month. As the infectivity at slaughter was calculated backward from the infectivity at clinical onset assuming an exponential decrease, the larger doubling time generated a higher level of infectivity long before clinical onset. Concerning the impact of prolonged incubation period, the assumed distribution of the incubation period was shifted by 0.5,

Table 24.1 Detection probability of the BSE-infected cattle at slaughterhouse with different testing strategies

Testing	Detection probability	Ratio
All cattle	20.3%	Base
Over 20 mo	20.1%	0.99
Over 24 mo	19.5%	0.96
Over 30 mo	15.5%	0.77

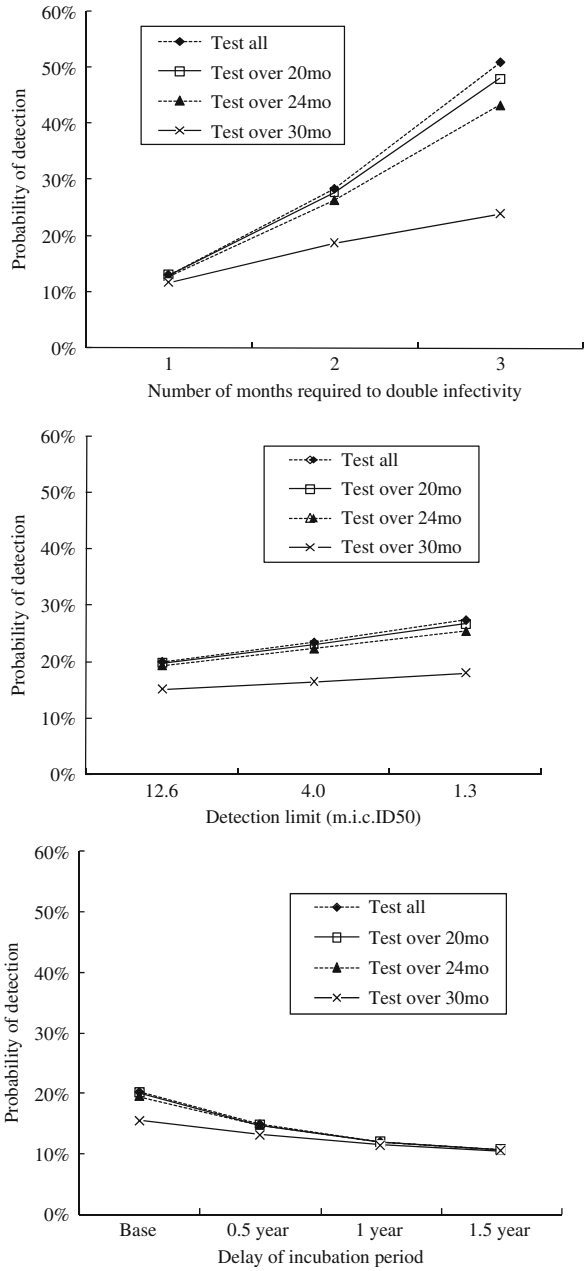

Fig. 24.6 The result of sensitivity analysis

1.0, and 1.5 years in order to express the delay in clinical onset. Delaying clinical onset reduced the probability of detection for all testing schemes, while the degree of reduction was smaller when cattle over 30 months are tested. This is because the longer incubation period resulted in lower infectivity at the time of slaughter. The effects of alternative values on detection limits for the screening tests were also analyzed. Lowering the detection limit slightly improved the probability of detection. However, the impact on the probability of detection was rather limited.

Simulation-Based Epidemiological Modeling Approach

We briefly introduce another approach that performs an epidemiological assessment by the use of a simulation model (Yamamoto et al., 2008). The back-calculation method usually uses mathematical equations to describe the model for calculating the number of infections in the past. In this approach, the simulation model is used to backward estimate the number of BSE-infected cattle that could have been a source of infection to humans instead of mathematical models.

First, the dynamics of BSE-infected animals in the cattle population are modeled by birth cohort, taking account of the coverage of the BSE surveillances currently in place. This individual-based simulation model generates the year of death and the final deposition of BSE-infected cattle, such as death on farm and detection at slaughter. The final dispositions of BSE-infected cows can be described and categorized as shown in Table 24.2. Parameters such as age at infection, incubation period, and age at slaughter/death are incorporated as probability distributions to generate stochastic effects. As a result, some infected cattle die before disease onset, while others live long enough to exhibit clinical symptoms. The probability of being tested is influenced by the year when an infected animal is slaughtered or dies, because the intensity of surveillance is different in different years. Infected cattle are assumed to be detected by surveillance if the animal is in the last stage of incubation period.

The output of the simulation model from a large number of iterations, with various numbers of initial infected animals, is compared to the observed BSE cases by each birth cohort. Then, the value with the highest likelihood is obtained as the maximum likelihood estimator for the number of infected animals in the birth cohort. The estimated number of infected cattle was then used to deduce the year and cause of slaughter/death of these BSE-infected cattle.

Table 24.2 Classification of the final disposition of BSE-infected cattle

Items	Classification	
Died due to clinical onset of BSE ?	Yes	No
Slaughtered or died on farm ?	Slaughter	Die
Tested by surveillance ?	Yes	No
Detectable infectivity ?	Yes	No

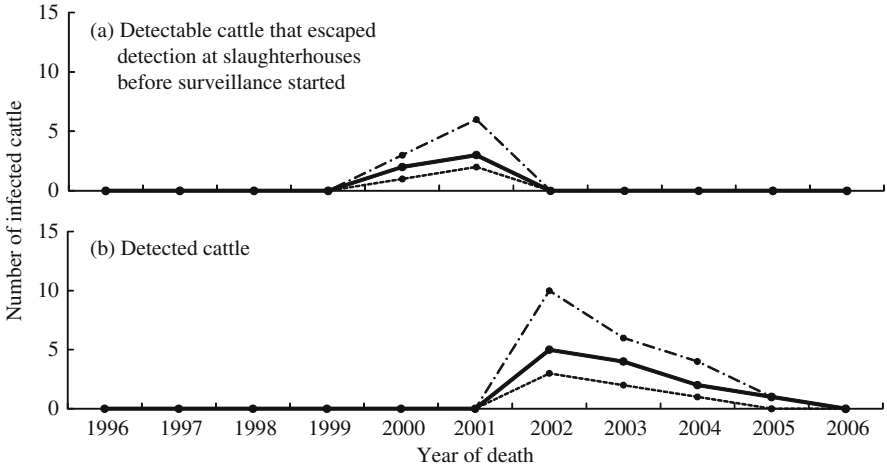

Fig. 24.7 The estimated number of the BSE-infected cattle which are in the last stage of incubation period

Figure 24.7 shows the estimated number of the BSE-infected cattle which are in the last stage of incubation period and are entered into the human food chain in Japan (Yamamoto et al., 2008). In the original study, a wide range of input parameters were tested in the sensitivity analysis.

Conclusions

Various quantitative approaches have been used for the food safety risk assessment of BSE. However, it is unavoidable that any type of model will be influenced by the uncertainty included in the input parameters. Therefore, sensitivity analysis is necessary to draw any variable indications from the model outputs. The results should be presented with these sensitivity analyses and underlying assumptions incorporated into the model. There is no definitive standard model developed for the risk assessment, and each approach has advantages and disadvantages. Therefore, a suitable approach should be chosen depending on the purpose of modeling. It is also noted that the model becomes outdated at a certain point in time. Any approach should be reviewed continuously according to updated knowledge on BSE.

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Chapter 25
Regulations on Meat Hygiene and Safety in the European Union

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Introduction

Meat safety concerns physical, chemical and biological aspects. With regard to these aspects it is important that the slaughterhouse and processing industry implement a HACCP programme.[1] By implementing such a programme all hazards, which could affect human safety, will have to be identified, monitored when considered as critical and eliminated, reduced or prevented. With regard to preventing physical hazards, a metal detector should be a regular piece of equipment in the meat processing industry.

With regard to the chemical aspects, residues and contaminants should be kept at as low a level as possible, but should certainly not exceed the maximum limits laid down in community legislation. To prevent residues and contaminants in meat, it is essential to follow good agricultural practice, which involves requirements regarding feeding and management, and to observe the correct withdrawal period following treatment of animals with veterinary medicines, a strict selection of raw materials, correct use of pesticides on grassland, to prevent the access of animals to toxins or environmental contaminants, etc.

With regard to the microbiological aspects meat inspection has focused traditionally on the detection of the major zoonotic diseases occurring in domestic animals, such as tuberculosis, cysticercosis, glanders, etc. In order to detect these diseases it was necessary to palpate and incise various parts of each slaughtered animal. However, these diseases have either been largely eradicated from herds kept under modern management conditions or

[1] HACCP stands for hazard analysis and critical control points.

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do not occur in the majority of the very young and generally healthy animals slaughtered nowadays. Moreover, it has been shown that meat inspection is in some specific cases not the most sensitive way to detect infestation. For example, it has been shown that in a sero-epidemiological study of *Taenia saginata* cysticercosis in cattle presented for slaughter in Belgium, the prevalence of bovine cysticercosis was more than 10 times higher with the antigen detection ELISA than by classical meat inspection (Dorny et al., 2000). Furthermore, micro-organisms that are of increasing zoonotic importance in modern animal husbandry systems, like salmonella and campylobacter, are readily transmitted from one carcase to the next by the various manipulations required to be performed during the traditional meat inspection procedures. Modern meat inspection should be based on risk assessment and should prevent cross-contamination in the slaughter hall (Berends, 1998). In addition, meat inspection can be improved by imposing stricter hygiene measures at the farm level and by requiring the farm operator to send relevant management and health information to the slaughterhouse for those animals that are to be slaughtered in the next 24 hours. These principles have been introduced in the new hygiene-related legislation introduced in the EU in recent years. The legislation will be explained in detail and related legislative aspects will be mentioned.

Reasons for Revising the Regulatory Aspects in the EU

In the 1990s consumer confidence was severely undermined due to a number of food contamination scares, such as the occurrence of bovine spongiform encephalitis in cattle initially in the United Kingdom and later in many other countries (Schreuder, 1994) and the detection of polychlorinated biphenyls and dioxins in animal feed in Belgium (Bernard et al., 1999). As a result, the Commission adopted the White Paper on Food Safety in 2000.[2] This ambitious programme contains a number of recommendations aimed at increasing food safety, improving the traceability of food products and regaining consumer confidence in the food industry. To this end a package of proposals for new legislation on food and feed has been prepared with the following characteristics: to complete and update the legislation; to improve official controls and ensure their efficient implementation. The package consists of a number of elements: the general food law, the hygiene package, a comprehensive set of requirements for the official controls by the competent authorities and related pieces of legislation.

[2] http://ec.europa.eu/food/food/intro/white_paper_en.htm

General Food Law

The objective of the general food law (Regulation (EC) No. 178/2002[3] of the European Parliament and of the Council) is to create a framework ensuring a universal consistency between all food and feed-related legislation. It lays down guiding principles and establishes common definitions, such as for "primary production", "food business operator", "retail", "risk" and "hazard". It aims at bringing together various related aspects of community legislation by including safety aspects and also protection of consumer interests, by covering all foods and by including both national and community levels. Furthermore, the Regulation puts the overall responsibility for producing safe food on the food business operator. It requires the food business operator to have a system in place enabling them to identify the supplier(s) of the raw materials and the immediate customer(s) of their products in order to ensure traceability. Other issues that are covered within this Regulation are the principles of risk analysis, withdrawal of food from the market by the food business operator if safety is at stake and the precautionary principle, which enables the adoption of provisional risk management measures as long as scientific uncertainty persists. Finally, it lays down the principles and requirements for the rapid alert system for food and feed (RASFF) and for the establishment of the European Food Safety Authority (EFSA). The RASFF is a network involving Member States, the Commission and EFSA. Whenever a member of the network has any information relating to the existence of a serious direct or indirect risk to human health, this information is immediately notified to the Commission under the RASFF. The Commission immediately transmits the information to the other members of the network, which should take corrective measures, if applicable. EFSA, based in Parma, Italy, is in charge of risk assessment and related risk communication. On the basis of the risk assessment, the legislators and decision-makers within the Commission and the Member States will develop and implement risk management measures and will communicate these measures accordingly.

Legislation on Food Hygiene

The "hygiene package" consists of a total of five legislative parts, of which four were adopted in April 2004 and provided the Member States and the stakeholders with a preparatory period of 18 months before becoming applicable with effect from 1 January 2006. The hygiene package puts the responsibility for producing safe food on the food business operator, while the competent authority of the Member State verifies correct implementation of the new rules.

[3] OJ L 31, 1.2.2002, p. 1. Regulation as last amended by Commission Regulation 202/2008 (OJ L 60, 5.3.2008, p. 17).

Production should be based on good hygienic practice and the HACCP principles and products are subject to microbiological criteria and temperature limits. The legislative texts deal with a variety of food types and cover the entire food chain ("from stable to table"). Two of the Regulations apply directly to food business operators:

- Regulation (EC) No. 852/2004 of the European Parliament and of the Council on the hygiene of foodstuffs.[4]
- Regulation (EC) No. 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin.[5]

Regulation (EC) No. 852/2004

Regulation (EC) No. 852/2004 lays down general hygiene requirements to be respected by food businesses at all stages of the food chain including primary production. The Regulation does not apply to small quantities of primary products supplied directly by the producer to the final consumer or by the producers to local retail establishments directly supplying the final consumer. Examples of such products are vegetables, fruits, eggs and raw milk or products collected in the wild such as mushrooms and berries. All bee-keeping activities are also considered as primary production. However, fresh meat is not a primary product since it is obtained after slaughter.

The Regulation requires all food business operators to put in place, implement and maintain a permanent procedure based on the Hazard Analysis and Critical Control Point (HACCP) principles with the exception of those involved in primary production. Food hygiene is the result of the implementation by food businesses of prerequisite requirements (such as concerning infrastructure and equipment, pest control, water quality, personal hygiene) and procedures based on the HACCP principles. The prerequisite requirements provide the foundation for effective HACCP implementation and should be in place before a HACCP-based procedure is established. The prerequisite requirements to be respected are laid down in an annex to the Regulation. The Regulation allows the HACCP-based procedures to be implemented with flexibility so as to ensure that they can be applied in all situations including in small businesses. Guides to good practice for hygiene and for the application of the HACCP principles developed by the food business sectors themselves, either at national or at community level, should help businesses to implement HACCP-based procedures tailored to the characteristics of their production.

In addition, the Regulation requires food businesses to be registered with the competent authority. Such registration is a simple procedure whereby the

[4] OJ L 139, 30.4.2004, p. 1, corrected by OJ L 226, 25.6.2004, p. 3.

[5] OJ L 139, 30.4.2004, p. 55, corrected by OJ L 226, 25.6.2004, p. 22. Regulation as last amended by Commission Regulation (EC) No. 1243/2007 (OJ L 281, 25.10.2007, p. 8).

competent authority is informed about the address of the establishment and the activities carried out. Already existing registration systems used for other purposes (environmental, animal health or other administrative purposes) can be used.

Regulation (EC) No. 853/2004

Regulation (EC) No. 853/2004 is more specific than the previous one by laying down the hygiene requirements to be respected by food businesses handling food of animal origin such as meat, live bivalve molluscs, fishery products, raw milk and dairy products, eggs and egg products, frogs' legs and snails, collagen and gelatine at all stages of the food chain. The Regulation does not apply to retail, which for food hygiene purposes means all activities involving direct sale or supply of food of animal origin to the final consumer. In such cases Regulation (EC) 852/2004 will apply. Establishments handling food of animal origin for which the Regulation lays down requirements in an annex must be approved. Those establishments carrying out only primary production, transport operations, storage of products not requiring temperature-controlled storage conditions or retail operations are exempted from the approval procedure. However, some of the retailers, especially the larger ones, do need approval. Approval procedures involve an on-site visit by the competent authority to verify if the establishment fulfils all the requirements concerning infrastructure, layout, equipment and hygiene. One of the requirements for approval is that the HACCP programme has been validated and implemented correctly. By granting a conditional approval for 3 months (and up to a maximum of 6 months) the competent authority will be able to assess the correct implementation of the HACCP programme under working conditions.

Other Legislation as Part of the Hygiene Package

Council Directive 2002/99/EC

Council Directive 2002/99/EC[6] lays down the animal health rules governing the production, processing, distribution and introduction of products of animal origin for human consumption. In an annex to the Council Directive the diseases relevant to trade in products of animal origin and for which control measures have been introduced under community legislation are listed. Another annex lists the treatments that are necessary to eliminate certain animal health risks linked to meat.

[6] OJ L 18, 23.1.2003, p. 11.

Directive 2004/41/EC

Directive 2004/41/EC[7] of the European Parliament and of the Council repeals the old legislation, a total of 16 Directives. Each Directive dealt with a specific food item (there was a Directive for meat, fish, milk, minced meat, etc.). Regulation (EC) No. 852/2004 repeals an additional Directive. Consequently, the legislation has been transformed from the so-called vertical Directives into a more horizontal approach ("from farm to fork").

Legislation on Official Controls

This legislation is directed at the competent authorities and lays down the general principles to be respected for ensuring the official controls are objective and efficient. Furthermore, the legislation has been designed to promote a more risk-based approach to official controls.

Regulation (EC) No. 882/2004 (Official Feed and Food Controls)[8]

Regulation (EC) No. 882/2004 of the European Parliament and of the Council on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules is the result of a review of the existing community rules on the subject, which were adopted separately for the animal feed sector, the food sector and the veterinary sector. The Regulation covers the basic principles and the entire range of activities dealing with feed and food law, including animal health, animal welfare and in certain aspects plant health. It applies with effect from 1 January 2006, except for the provision on financing of official controls, which applies with effect from 1 January 2007.

As a consequence of the new rules, the Member States have to reorganise their official control systems so as to integrate controls at all stages of production and in all the concerned sectors, using the "farm to fork" principles. They have to submit and annually update a multi-annual national control plan for the implementation of feed and food legislation and to report annually on the implementation of that plan. The control plans and reports shall take into account guidelines published by the Commission as Commission Decision 2007/363/EC.[9]

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[7] OJ L 195, 2.6.2004, p. 12.

[8] OJ L 165, 30.4.2004, p. 1, corrected by OJ L 191, 28.5.2004, p. 1. Regulation as last amended by Council Regulation (EC) No. 301/2008 (OJ L 97, 9.4.2008, p. 85).

[9] OJ L 138, 30.5.2007, p. 24.

At present, community controls in the Member States and in third countries are organised largely on a sectoral basis and are related to the mandates the Commission has in different sectoral legislation. By means of this Regulation the community approach to controls will evolve. The role of the Food and Veterinary Office as part of the European Commission will be essentially based on audit with the main purpose of verifying the efficiency of the control systems in the Member States and auditing the compliance or equivalence of third country legislation and control systems with EU rules. The requirement for all Member States to submit a multi-annual national control plan will facilitate the carrying out of these audits. Account will also be taken of Member States' own audits and of their annual reports.

The Regulation provides for a set of general rules applicable to the official controls of all feed and food at any stage of production, processing and distribution, whether produced within the EU, exported to or imported from third countries. In addition to these rules, there are other specific control measures, which are important in order to maintain a high level of protection and therefore must be kept in place. This is, for example, the case for the specific veterinary control rules on imports of animals and food of animal origin or for the specific control rules for organic products. The Regulation provides the possibility to draw up a list of feed and food of non-animal origin, which shall be subject to an increased level of official controls at the point of entry.

Regulation (EC) No. 854/2004

Regulation (EC) No. 854/2004 of the European Parliament and of the Council lays down specific rules for the organisation of official controls on products of animal origin intended for human consumption.[10] The Regulation forms the third part of the "hygiene package" and deals, among other things, with the official control of animals sent for slaughter, official control with regard to fresh meat, fishery products, raw milk and dairy products and with procedures concerning imports. Modern meat inspection should be based on risk assessment and should prevent cross-contamination in the slaughter hall. In addition, meat inspection can be improved by imposing stricter hygiene measures at the farm level and by requiring the farm operator to send animals for slaughter in a clean state together with relevant management and health information called food chain information. These principles have been introduced in the Regulation.

[10] OJ L 139, 30.4.2004, p. 206, corrected by OJ L 226, 25.6.2004, p. 83. Regulation as last amended by Council Regulation (EC) No. 1791/2006 (OJ L 363, 20.12.2006, p. 1).

Implementing Measures of the Hygiene Package

Implementing Measures

A wide range of implementing measures have been adopted on the basis of the hygiene package as foreseen in Article 12 of Regulation (EC) 852/2004, Articles 9 and 11 of Regulation (EC) 853/2004 and Articles 16 and 18 of Regulation (EC) 854/2004.

The measures laid down in Commission Regulation (EC) 2074/2005[11] include provisions concerning food chain information, fishery products, recognised testing methods for detecting marine biotoxins, calcium content of mechanically separated meat, lists of establishments, model health certificates for a number of products (frogs' legs, snails, gelatine, collagen, fishing products and honey), a derogation for foods with traditional characteristics and a number of amendments to Regulations (EC) 853/2004 and (EC) 854/2004. The amendments rectify some minor details in the Regulations.

Transitional Arrangements

Transitional arrangements in respect of certain new provisions have been taken to permit a smooth change over from the old to the new regime. The principle of granting transitional arrangements was agreed by the European Parliament and the Council through Article 12 of Regulation (EC) No. 852/2004, Article 9 of Regulation (EC) No. 853/2004 and Article 16 of Regulation (EC) No. 854/2004.

The measures laid down in Commission Regulation (EC) No. 2076/2005[12] include provisions concerning stocks of food of animal origin, placing of food of animal origin on national markets, materials bearing pre-printed health or identification marks, marking equipment, health import conditions, food chain information, composition criteria for minced meat, use of clean water, raw milk and dairy products, eggs and egg products, training of slaughterhouse staff, certification of establishments, accreditation of laboratories carrying out official controls and some amendments to Regulations (EC) 853/2004 and (EC) 854/2004. The transitional period is in some cases 2 years, but in most cases 4 years ending on 31 December 2009.

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[11] OJ L 338, 22.12.2005, p. 27 corrected by OJ L 320, 18.11.2006, 13. Regulation as last amended by Commission Regulation (EC) No. 1244/2007 (OJ L 281, 25.10. 2007, p. 12).

[12] OJ L 338, 22.12.2005, p. 83. Regulation as last amended by Commission Regulation (EC) No. 439/2008 (OJ L 132, 22.5.2008, p. 16).

Examination of Meat for Trichinella Parasites

The adoption of Directive 2004/41/EC on 21 April 2004 by the European Parliament and the Council resulted in the repeal of Council Directive 77/96/EEC, which specified in detail the examination of carcases of swine, horses and other susceptible species for *Trichinella*. Commission Regulation (EC) No. 2075/2005[13] has retained many elements from the previous legislation such as the sampling procedure, the various examination techniques in the laboratory and the derogations granted. However, at the same time the Commission Regulation has introduced a number of new elements to increase food safety for the consumer and facilitate the sampling procedure for those establishments where the parasite has not been encountered for a long time. The new elements are the following:

- A larger amount of sample has to be collected and examined from those animal species that pose the greatest risk for infecting humans, mainly horses and wild boar.
- Freezing is no longer allowed to replace the examination of horsemeat, because in this host certain *Trichinella* species such as *T. spiralis*, *T. pseudospiralis* and *T. britovi* can survive freezing temperatures.
- The use of the trichinoscopic method for examining meat samples is no longer allowed, because it fails to detect *T. pseudospiralis*. This species does not elicit the formation of a collagen capsule in the muscle tissues and, therefore, is very difficult to detect in large animals using this method. A transitional arrangement for 4 years will give the competent authority the possibility to switch to a more reliable examination method. A number of additional requirements have to be applied whenever the trichinoscopic method is used.
- The most important regulatory change is the introduction of *Trichinella*-free holdings or category of holdings or regions having a negligible prevalence. The competent authority can recognise a holding as free from *Trichinella* following an on-site inspection. Animals coming from a *Trichinella*-free holding or from a region with a negligible *Trichinella* risk are exempted from examination for *Trichinella*. The derogation applies only to fattening pigs. Inspection procedures can be very much simplified when the competent authority decides to recognise a category of holdings as free from *Trichinella*. Finally, the Regulation provides the possibility for a Member State to declare a region as having a negligible prevalence for *Trichinella*. Third countries will be able to apply the derogation of declaring a holding as free from *Trichinella* as well.

[13] OJ L 338, 22.12.2005, p. 60. Regulation as last amended by Commission Regulation (EC) No. 1245/2007 (OJ L 281, 25.10.2007, p. 19).

Regulation on Microbiological Criteria for Foodstuffs

Previously existing microbiological criteria were reviewed taking into account recent developments in food microbiology and scientific advice from the European Food Safety Authority (EFSA). Commission Regulation (EC) No. 2073/2005[14] revised these criteria and introduced additional ones. The main objectives of the Commission Regulation are to ensure a high level of consumer protection with regard to food safety and to harmonise the microbiological criteria in the Member States, thus facilitating international trade. In particular, the target of the commission regulation is to reduce the number of *Salmonella*, *Listeria* and *Enterobacter sakazakii* cases in humans. A main component of the Regulation is to set two different types of criteria for foodstuffs, which need to be complied with by the food business operator:

- A food safety criterion defining safety of a product or a batch applicable to products placed on the market
- A process hygiene criterion indicating the correct functioning of the manufacturing process

Food safety criteria have been laid down for certain micro-organisms which are common causes of food-borne diseases in humans, such as *Salmonella*, *Listeria monocytogenes*, *E. sakazakii*, staphylococcal enterotoxins and histamine. If food safety criteria are exceeded, the batch has to be withdrawn from the market. Food safety criteria have been set for the following combinations of food category/micro-organisms:

- A *L. monocytogenes* criterion for all ready-to-eat foods
- A *Salmonella* criterion for certain ready-to-eat foods, minced meat, meat preparations and certain meat products
- A criterion for staphylococcal enterotoxins in certain types of cheeses and milk powder
- An *E. sakazakii* criterion for dried infant formulae
- An *Escherichia coli* criterion in live bivalve molluscs
- A histamine criterion for certain fishery products

In addition, the Commission Regulation includes process hygiene criteria, such as Enterobacteriaceae and *Salmonella* in carcases of slaughtered animals, *Staphylococci* in certain types of cheese, *E. coli* in pre-cut fruit and vegetables.

The sampling frequency is stipulated for a few criteria, for example, for *Salmonella* in minced meat and carcases. In other cases the food business operators have to decide the sampling frequency on a case-by-case basis taking into account the risk related to their products. Although the Commission Regulation is directed at food business operators, competent authorities may,

[14] OJ L 338, 22.12.2005, p. 1. Regulation as last amended by Commission Regulation (EC) No. 1441/2007 (OJ L 322, 7.12.2007, p. 12).

for various reasons, take samples to ensure that the criteria laid down are met. In the absence of community microbiological criteria the evaluation of the food can be done in accordance with Article 14 of Regulation (EC) No. 178/2002, which provides that unsafe food must not be placed on the market.

Guidance Documents for the Legislation on Food Hygiene

A number of documents have been prepared to give guidance on the implementation of the food hygiene requirements and related subjects. The documents aim to assist the food business operators and the competent authorities of the Member States. The guidance documents are not formal acts of legislation, but the Commission will defend where necessary the consensus laid down in these documents. Most of the documents (with an *) have been placed on the DG SANCO Internet site:[15]

- Guidance document on the implementation of the main General Food Law requirements*
- Guidance document on Regulation (EC) No. 852/2004*
- Guidance document on Regulation (EC) No. 853/2004*
- Guidance document on the implementation of HACCP and facilitation of the implementation of the HACCP principles in certain food businesses*
- Guidance document on community guides to good practice*
- Practical guide on food contact materials*
- Guidance documents on import requirements*
- Guidance document on the preparation of multi-annual control plans as laid down in Regulation (EC) No. 882/2004 (published as Commission Decision 2007/363/EC[16])
- Guidance document laying down criteria for the conduct of audits (published as Commission Decision 2006/677/EC[17])
- Guidance document on official controls, under Regulation (EC) No. 882/2004, concerning microbiological sampling and testing of foodstuffs*.

Related Food Safety Legislation

Legislation on Transmissible Spongiform Encephalopathies

The recognition of the first cases of bovine spongiform encephalopathy (BSE) in the mid-1980s and the first diagnoses of variant Creutzfeldt–Jakob disease (vCJD) in humans in 1996 together with the causal link between BSE and vCJD

[15] http://ec.europa.eu/food/food/index_en.htm

[16] OJ L 138, 30.5.2007, p. 24.

[17] OJ L 278, 10.10.2006, p. 15.

led to one of the major crises which ever affected the feed and food sectors. The key piece of legislation to protect human and animal health from the risk of BSE and other transmissible spongiform encephalopathies (TSEs) is Regulation (EC) No. 999/2001[18] of the European Parliament and of the Council which lays down rules for the prevention, control and eradication of certain TSEs and is commonly known as the "TSE Regulation". The TSE Regulation provides measures targeting all animal and public health risks resulting from all animal TSE and governing the entire chain of production and placing on the market of live animals and products of animal origin. It consolidates much of the existing legislation on BSE or TSE, including rules for the monitoring of TSE in bovine, ovine and caprine animals and prohibitions concerning animal feeding. It also introduces new legislation for areas such as eradication of TSE as well as trade rules covering the domestic market, intra-community trade, import and export. Furthermore, it provides for the procedure, criteria and categories for the classification of countries according to BSE status. This very comprehensive framework is constantly evaluated through scientific review. The removal of the so-called specified risk material is one of the most important measures to protect the health of consumers against the risk of BSE. Specified risk materials are defined as the animal tissues being most at risk of harbouring the TSE agent. In order to prevent any recycling of possible BSE agent, these tissues are collected and completely destroyed through incineration. Recently the Commission produced a road map on the TSE strategy,[19] which outlines possible amendments of certain measures in the short, medium and long term without endangering the health of the consumer or the policy of eradicating BSE.

Legislation on Animal By-Products

Regulation (EC) No. 1774/2002[20] of the European Parliament and of the Council lays down health rules for the collection, transport, storage, handling, processing and use or disposal of all animal by-products (ABPs) not intended for human consumption. It completes the rules laid down in Regulation (EC) No. 852/2004 on food waste. Its purpose is to prevent by-products from presenting a risk to animal or public health. To that end, it distinguishes three different categories of ABPs, based on risk. Category 1 material has the highest risk and is usually incinerated. Category 2 is less risky material and can not only be incinerated but also be composted or used for biogas production. Category 3 material can be used for animal feed under certain conditions. The last

[18] OJ L 147, 31.5.2001, p. 1. Regulation as last amended by Commission Regulation (EC) No. 357/2008 (OJ L 111, 23.4.2008, p. 3).

[19] The TSE road map, Brussels, 15 July 2005: http://ec.europa.eu/food/food/biosafety/bse/index_en.htm

[20] OJ L 273, 10.10.2002, p. 1.

category includes parts of slaughtered animals that have been found fit for human consumption, but are not for one reason or another intended for human consumption.

Legislation on Residues

Residues are substances that can occur in foodstuffs as a side effect of using veterinary medicines or phyto-sanitary products. They are unwanted traces of medicines or plant protection products or derivatives thereof which remain in the final product.[21] Member States need to adopt and implement every year a plan to monitor live animals and products thereof, including meat, for residues of prohibited substances (for example, hormonal substances for fattening purposes) or for substances permitted below a certain threshold, the so-called maximum residue limit (MRL). The latter group of substances includes veterinary medicinal products, pesticides and environmental contaminants. Details of the substances involved and of the residue monitoring plan can be found in Council Directive 96/22/EC[22] and Council Directive 96/23/EC[23] and their amendments. The aim of the national residue monitoring plan is to ensure that permitted levels are not exceeded and that forbidden substances are not present in food products.

Legislation on Contaminants

Contaminants are substances that can unintentionally enter food during the various stages of its production, packaging, transport or holding or as a result of environmental contamination. Council Regulation (EC) No. 315/93[24] lays down the basic principles to minimise contaminants in food, while Commission Regulation (EC) No. 1881/2006[25] sets maximum levels for certain contaminants in foodstuffs. For fresh meat maximum levels have been laid down with regard to heavy metals, dioxins and polychlorinated biphenyls (PCBs).

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[21] Questions and answers on residues and contaminants in foodstuffs (February 2003).

[22] OJ L 125, 23.5.1996, p. 3. Directive as last amended by Directive 2003/74/EC (OJ L 262, 14.10. 2003, p. 17).

[23] OJ L 125, 23.5.1996, p. 10. Directive as last amended by Council Directive 2006/104/EC (OJ L 363, 20.12.2006, p. 352).

[24] OJ L 37, 13.2.1993, p. 1.

[25] OJ L 364, 20.12.2006, p. 5.

Other Legislation

As soon as meat is being processed additional legislation will start to apply, such as rules on food additives (substances added intentionally to foodstuffs to perform certain technological functions), biocides, food contact materials and labelling requirements. Animal health requirements will apply to the live animals that are going to be slaughtered, while welfare requirements will apply to the management of animals on the farm, the transport of animals to the slaughterhouse and the killing of animals at the slaughterhouse.

International Aspects

Where international standards exist, they have been taken into consideration in the development or adaptation of the food safety legislation. This applies to standards developed by the Codex Alimentarius Commission, which has been created by the Food and Agriculture Organization and the World Health Organization to develop food standards, guidelines and related texts such as codes of practice. It also applies to standards related to animal health and animal welfare developed by the World Organisation for Animal health (OIE). Similarly, ISO and CEN standards have been incorporated in the legislation as analytical reference methods as far as possible (as can be seen for example in Commission Regulation (EC) No. 2073/2005).

Future Legislative Work

Treatment to Remove Surface Contamination

Article 3(2) of Regulation (EC) No. 853/2004 provides a legal basis to permit substances other than potable water to remove surface contamination from products of animal origin. Such a legal basis did not exist in the previous legislation (Directive 64/433 for red meat, Directive 71/118 for poultry meat, other Directives used to cross-reference to the former Directive mentioned), but is available now that Regulation (EC) No. 853/2004 is applicable.

With the adoption of the hygiene package and the introduction of the HACCP principles in the entire food chain, establishments are obliged to improve their hygiene and processing procedures. Under such circumstances the use of substances to remove surface contamination of food of animal origin can be reconsidered. It is essential that a fully integrated control programme is applied throughout the entire food chain including reduction of pathogens in water and in feed, on farms, during transport and in the processing plant. Treatment to remove surface contamination might constitute an additional element in further reducing the number of pathogens, especially with regard

to *Salmonella* and *Campylobacter*, provided an integrated control strategy is applied throughout the entire food chain. A draft implementing Commission Regulation to permit under specified circumstances the use of four substances for the removal of surface contamination of poultry meat is currently in preparation and is being discussed.

Risk-Based Meat Inspection

Meat inspection has focused traditionally on the detection of the major zoonotic diseases. As stated in the introduction these diseases have either been largely eradicated from herds kept under modern management conditions or do not occur in the majority of the very young and generally healthy animals slaughtered nowadays. In order to prevent cross-contamination of subsequent carcases with micro-organisms of importance in modern animal husbandry systems, like *Salmonella* and *Campylobacter*, inspection procedures without incision or palpation will have to be introduced. A detailed visual inspection without any incision or palpation of slaughter animals might be sufficient to ensure food safety. However, under these circumstances it will be necessary to take efficient preventive measures during the rearing of the animals and to provide sufficient information to the slaughterhouse on the life history of the animals. Commission Regulation (EC) No. 1244/2007[26] lays down detailed requirements for a risk-based meat inspection of fattening pigs and young ruminants and lays down the conditions for the competent authority to apply such a system if appropriate.

Report to the European Parliament and the Council

The Commission will have to submit a report to the European Parliament and the Council not later than 20 May 2009. The report shall review the experience gained with the application of the hygiene package and can recommend changes, amendments or improvements to facilitate implementation of the new rules.

Implications of the New Legislation for the Official Veterinarian

The official veterinarian (OV) is the veterinarian appointed by the competent authority and with the qualifications as described in Chapter IV, Section III, Annex I to Regulation (EC) No. 854/2004. The tasks of the OV have shifted slightly due to the new hygiene legislation. First, the food business operator (for

[26] OJ L 281, 25.10.2007, p. 12.

example, the slaughterhouse operator) is responsible for producing safe food and the duties of the competent authority have been limited to verification of compliance with the legal requirements. Second, the duty of the OV to supervise the slaughter process will be expanded with additional tasks such as conducting audits (as described in Commission Decision 2006/677/EC), HACCP verifications, assessment of food chain information and the application of risk-based meat inspection. In order to fulfil these new tasks properly it will be necessary for the OV to engage in continuing education activities and for the competent authority to ensure that all of its staff receives regular additional training.

Implications of the New Legislation for the Private Veterinarian

In this case the farmer will be responsible for producing safe food and the private veterinarian can serve as a valuable advisor. In order to ensure food safety it is important that the farmer follows the general hygiene provisions (as described in Annex I to Regulation (EC) No. 852/2004). In addition, the farmer is required to keep records of the origin of animal feedstuffs, the use of veterinary medicinal products, the results of any analyses carried out on relevant samples and the use of plant protection products, if applicable. However, the farmer is not required to implement procedures based on the HACCP principles. Once the farmer decides to send animals for slaughter, it will be necessary to ensure that the animals are cleaned beforehand and to forward food chain information to the slaughterhouse operator. The private veterinarian can be of assistance with the latter requirement especially if animal diseases, treatments or laboratory results need to be mentioned or interpreted. Moreover, the private veterinarian can assist the farmer in the interpretation of the reports containing slaughter findings that the slaughterhouse operator should be sending back on a regular basis. As far as clean animals are concerned, a scoring method as developed for cattle by the British Food Standards Agency as part of the "clean livestock policy" could be of some assistance.[27] In addition, the private veterinarian can assist the farmer to fulfil the requirements for risk-based meat inspection (as described in Annex II to Commission Regulation (EC) No. 1244/2007) and the requirements for the official recognition of holdings as free from *Trichinella* (as described in Chapter I, Annex IV to Commission Regulation (EC) No. 2075/2005). Finally, the private veterinarian can assist the farmer with ensuring a continuous delivery of raw milk from the production holding by complying with the criteria with regard to plate counts and somatic cell counts (as described in Chapter I, Section IX, Annex III to Regulation (EC) No. 853/2004).

[27] See FSA web site: http://www.food.gov.uk/foodindustry/farmingfood/cleancattleandmeatsafety.

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Chapter 26
Regulations on Meat Hygiene in the USA

Robert (Skip) A. Seward

Introduction

Regulations on meat hygiene in the United States of America (US) stem from the Federal Meat Inspection Act (FMIA, 21 USC §§ 601 *et. seq.*), promulgated in 1906, that gives the US Secretary of Agriculture (the Secretary) the power to oversee the conversion of livestock into meat products. The FMIA is reviewed herein to provide a background for discussion on how the US Department of Agriculture (USDA) and its departments, particularly the Food Safety and Inspection Service (FSIS), control and regulate the meat industry. This chapter discusses regulations that pertain to meat, herein meant to mean beef, veal, and pork, and does not specifically address poultry, although the regulations for poultry slaughter and processing are in many ways similar to those for meat and meat food products.

The FMIA addresses numerous issues important to meat hygiene, including animal slaughter, fabrication and processing, sanitary conditions, inspection requirements, regulations promulgated to support the FMIA, labeling, packaging, transportation, consequences of failure, and definitions of adulteration and misbranding. The USDA and FSIS, the departments that enforce the FMIA and its regulations, use many other instruments to enforce the FMIA, including rules, directives, notices, and performance standards. These instruments, and their impact, will be discussed herein to describe how FSIS leverages its tools to regulate meat hygiene.

Background on the US Meat Industry

In 2007, about 302 million US residents shared resources with approximately 97 million head of cattle and calves, over 6 million sheep and lambs, and over 62 million hogs. These animals are managed on 971,400 cattle operations,

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65,540 hog operations, and 69,090 sheep operations. Beef cattle feedlots numbered 2,165 in 2006, with nearly 88% managing 1,000–15,999 cattle in a given feedlot; 5.8% of the feedlots had more than 32,000 cattle in a given feedlot. Of the 65,540 hog operations, 54% had more than 5,000 head in each operation. In 2006, 33,698,000 cattle were slaughtered to produce 11,910 million kg of beef; 711,000 calves were slaughtered to yield 70.3 million kg of veal; 2,698,000 sheep and lambs were slaughtered to produce 86.2 million kg of lamb and mutton; and 104,737,000 hogs were slaughtered to yield 9,559 million kg of pork. Over 92% of slaughtered livestock and meat production (kg) occurred in USDA-inspected establishments.

Cash receipts in 2006 from cattle and calves, hogs, and sheep and lambs were over $49 billion, $14 billion, and $480 million, respectively, for a total of over $63 billion, or about 26% of all cash receipts from farming operations in the US. Per capita US beef consumption at retail in 2006 was 29.8 kg; veal was 0.23 kg; pork was 22.4 kg; and lamb and mutton consumption was 0.45 kg, for a total red meat retail US per capita consumption of 52.9 kg. This compares to 2006 per capita US chicken and turkey consumption of 27.9 and 6.0 kg, respectively. US 2006 per capita expenditures for beef, pork, chicken, and turkey were $260.63, $139.02, $135.68, and $18.92, respectively. In 2006 US consumers spent 1.7% of their 2006 disposable personal income (mean value $32,115) on beef and poultry and 15.3% on all food expenditures.

The top five beef slaughter operations in 2007 were Tyson Foods (31,175 head/day), Cargill Meat Solutions (25,850/day), JBS Swift (15,800/day), National Beef Packing (7,600/day), and American Foods Group (5,200/day). Tyson Foods, Cargill Meat Solutions, JBS Swift, and National Beef Packing represent 70% of the total US daily steer and heifer slaughter capacity. American Foods Group, Cargill Meat Solutions, XL Beef, and Smithfield Foods represent 56% of the total US daily cow slaughter capacity. The top five hog slaughter operations in 2007 were Smithfield Foods (114,300 head/day), Tyson Foods (67,600/day), JBS Swift (39,500/day), Hormel Foods (37,000/day), and Cargill Meat Solutions (29,500/day); these five companies represent 67% of the total US daily hog slaughter capacity.

In 2006, cattle, calves, hogs, and sheep and lambs were slaughtered in 636, 238, 614, and 484 USDA-inspected establishments, respectively. Of the 636 USDA-inspected cattle slaughter plants, 9.6% slaughter 97.3% of the cattle. Nebraska, Kansas, and Texas were the three states within the US where the highest percentages of cattle were slaughtered. Of the 614 USDA-inspected hog slaughter plants, 8.6% slaughter 96.9% of the hogs. Iowa, North Carolina, Minnesota, and Illinois were the four states within the US where the highest percentages of hogs were slaughtered.

Of the 33,698,000 beef animals slaughtered in 2006, 82.4% were steers and heifers, 16.1% were cows, and 1.5% were bulls and stags. In 2006, 2.6, 51.7, and 32.7% of graded beef had a "prime," "choice," and "select" quality grade, respectively. In 2006, margins for beef packers and feedlot operations were $2.25 and a negative $49.72 per head, respectively. For pork processors, the

2006 margin per head was $0.77. The number one meat company ranked by 2006 sales was Tyson Foods with $26 billion in sales, stemming from 114,000 employees working at 123 plants. Cargill Meat Solutions was number two with $15 billion from 33,000 employees at 23 plants.

In 2006, imports of beef and veal into the US equaled 11.7% of US beef and veal production and cost approximately $3.2 billion; exports equaled 4.3% of production and were valued at over $1.5 billion. In 2006, imports of pork into the US equaled 4.7% of US pork production and cost nearly $1.2 billion; exports equaled 14.2% of production and were valued at over $2.5 billion. In 2006, imports of total red meat into the US equaled 8.9% of US total red meat production; and exports equaled 8.7% of production. These red meat imports were primarily fresh and frozen beef (about 902,000 metric tons), but also included fresh or frozen pork (over 342,000 metric tons), and processed and canned beef and pork (over 101,000 and nearly 76,000 metric tons, respectively).

Consumer Views on Meat Hygiene in the US

The US meat industry is considered by many to be a highly efficient producer of safe, affordable meat. However, the US media generally reports only negative stories related to meat production such as recalls or outbreaks where meat has been identified as a possible vehicle. If the stories become repetitive and exaggerated, consumer confidence and purchasing behaviors can become affected negatively. For example, according to the 2007 annual survey by the Food Marketing Institute, consumer confidence in food of all types purchased at restaurants and grocery stores declined by 16% over the preceding year (FMI, 2007), possibly because of highly publicized outbreaks and recalls associated with a variety of foods from peanut butter to fresh spinach that occurred in 2007.

At the beginning of 2007, the American Meat Institute (AMI) conducted an industry image survey to assess current public opinion of the meat and poultry packing and processing industry. The surveys consisted of two Chicago, Illinois-based focus groups and an Internet survey. Some of the key findings of this research included the following:

- About 90% of consumers agreed that meat products manufactured in the US are among the safest in the world.
- About 74% agreed that US meat products are among the most affordable in the world.
- Nearly 60% agreed that meat and poultry animals are treated humanely.
- When asked the open-ended question about what prevents them from eating red meat more often, 24% cited issues related to health and diet.
- About 50% remembered hearing about specific incidents regarding food safety problems with meat in the last year.
- One-third said that food safety issues have prevented them from purchasing meat or poultry in the past.

In response to the open-ended question about what comes to mind when thinking about the meat packing and processing industries, 26% said cleanliness and sanitation and 11% said safety and handling of meat. When asked to rate how favorably they viewed the meat industry on a scale of 1–10, the mean response was 7. When asked if they knew that USDA inspectors were constantly in meat plants and how that would affect their feelings toward the industry, 72% responded that it would make them feel more positive.

The Federal Meat Inspection Act of the US

The regulations for meat and poultry hygiene in the US are stated in the FMIA and the Poultry Products Inspection Act (PPIA, 21 USC §§ 451 *et. seq.*) with subsequent amendments and were initially established in 1906 and 1957, respectively. These acts were promulgated to ensure that consumers would find meat and poultry products wholesome, not adulterated, and properly marked, labeled, and packaged. The Secretary is instructed to use inspectors to examine and inspect all cattle, sheep, swine, goats, horses, mules, and other equines before they enter any slaughter, packing, meat canning, rendering or similar establishment, and to ensure humane methods of slaughter are used. Inspectors are to examine and inspect all carcasses from these animals that are used in commerce via a postmortem examination and inspection. Carcasses and parts thereof of all such animals found to be not adulterated are marked, stamped, tagged, or labeled as "inspected and passed," some based on standards of identity. If marked as "inspected and condemned," carcasses and parts thereof are destroyed for food purposes by the establishment in the presence of an inspector. The Secretary must ensure that the labeling of meat products is not false or misleading.

The FMIA authorizes the Secretary to prescribe the rules and regulations of sanitation for establishments producing meat products and authorizes the examination and inspection of slaughter and meat processing establishments anytime during any day. The FMIA also requires the same inspection for live animals, carcasses, or meat derived from carcasses destined for export and authorizes the Secretary to appoint inspectors to issue official certificates stating that the conditions of animals, carcasses, or meat derived from carcasses are acceptable for export. Similarly, all imported carcasses, parts of carcasses, meat, or meat food products of cattle, sheep, swine, goats, horses, mules, or other equines which are capable of use as human food, as well as the foreign establishments producing these products, must comply with all the inspection, sanitation, quality, species verification, residue, building and construction standards, and all other provisions of the FMIA and regulations applicable to such articles in commerce, and the production facilities in which such articles are produced, within the US. All meat products sold in the US must be derived from livestock that were slaughtered and handled in accordance with

the Humane Slaughter Act (7 USC §§ 1901 *et. seq.*), the Animal Welfare Act (7 USC §§ 2131 *et. seq.*), the FMIA, and all associated regulations.

The FMIA does not apply to the slaughter of animals raised by individuals for consumption by themselves or their household, and nonpaying guests and employees, or to the custom slaughter of animals, including game animals, delivered by the owner for their own use as long as such operations are separate from regulated operations and operated in a sanitary manner so as not to produce adulterated or misbranded products.

The FMIA also states that the Secretary may regulate conditions under which carcasses, parts of carcasses, meat, and meat food products of cattle, sheep, swine, goats, horses, mules, or other equines, capable of use as human food, shall be stored or otherwise handled by any person, firm, or corporation engaged in the business of buying, selling, freezing, storing, or transporting or importing such items. However, the regulations do not apply to the storage or handling of such items at retail stores.

The FMIA also requires records be kept that will fully and correctly disclose all transactions involved in operations that involve slaughter, processing, packaging, or labeling of animals or parts thereof for use as human food or animal feed. Such places of business can be accessed and examined by duly authorized representatives of the Secretary; inventory can be examined; and records can be reviewed and copied. All persons, firms, or corporations engaged in businesses that include brokerage of meat, rendering, manufacturing of animal food, or manufacturing of carcasses, or parts or products of the carcasses must register with the Secretary the name and the address of each place of business at which, and all trade names under which, such business is conducted. The FMIA states that USDA will assist state meat inspection agencies that may, at their discretion and in lieu of USDA inspection, manage intrastate meat commerce to establish equivalency. The Secretary has the responsibility to ensure that meat items produced under state inspection are neither adulterated nor misbranded.

The US district courts, the District Court of Guam, the District Court of the Virgin Islands, the highest court of American Samoa, and the US courts of the other territories are vested with jurisdiction to enforce the FMIA. The FMIA also defines the financial penalties and imprisonment terms for individuals who forcibly assault, resist, oppose, impede, and intimidate inspection staff or others officially charged with executing the FMIA and for persons, firms, or corporations who violate any provision of the FMIA.

Because the FMIA provides authority to the Secretary to prevent adulterated and misbranded meat products from reaching consumers, it is appropriate to provide definitions for these terms as written in the FMIA. The term "adulterated" applies to any carcass, part thereof, meat, or meat food product that

- bears or contains any poisonous or deleterious substance which may render it injurious to health; but in the case the substance is not an added substance, such article shall not be considered adulterated if the quantity of such substance does not ordinarily render the article injurious to health;

- bears or contains by reason of administration of any substance to the live animal or otherwise any added poisonous or added deleterious substance (other than one which is a pesticide chemical in or on a raw agricultural commodity, a food additive, or a color additive) which may, in the judgment of the Secretary, make such article unfit for human food;
- bears or contains a pesticide chemical, food additive, or color additive which is unsafe or used outside of permissible regulations;
- contains any filthy, putrid, or decomposed substance or is for any other reason found to be unsound, unhealthful, unwholesome, or otherwise unfit for human food;
- has been prepared, packed, or held under insanitary conditions whereby it may have been contaminated with filth or whereby it may have been rendered injurious to health;
- represents, in whole or in part, the product of an animal which has died otherwise than by slaughter;
- is held in a container that is composed, in whole or in part, of any poisonous or deleterious substance which may render the contents injurious to health;
- has been subjected to unapproved radiation; or
- has had any valuable constituent omitted, or has had any substitutions, or has had damage or inferiority concealed in any way, or has had a substance added so as to increase its bulk or weight, reduce its quality or strength, or make it appear better or of greater value than it is.

The term "misbranded" shall apply to any carcass, part thereof, meat, or meat food product under one or more of the following circumstances:

- if its labeling is false or misleading in any particular;
- if it is offered for sale under the name of another food;
- if it is an imitation of another food, unless the label bears the word "imitation," and immediately thereafter the name of the food imitated;
- if its container is so made, formed, or filled as to be misleading;
- if it is in a package or other container unless it bears a label showing the name and place of the manufacturer, packer, or distributor, an accurate statement of the quantity of the contents in terms of weight, measure, or numerical count within permissible variations;
- if any word, statement, or other information required by or under authority of the FMIA to appear on the label or other labeling is not prominently placed thereon with such conspicuousness and in such terms as to render it likely to be read and understood by the ordinary individual under customary conditions of purchase and use;
- if it purports to be or is represented as a food for which a definition and standard of identity or composition has been prescribed by regulations unless it conforms to such definition and standard, and its label bears the name of the food specified in the definition and standard and, insofar as may be required by such regulations, the common names of optional ingredients present in such food;

- if it purports to be or is represented as a food for which a standard or standards of fill of container have been prescribed by regulations and it falls below the standard of fill of container applicable thereto, unless its label bears, in such manner and form as such regulations specify, a statement that it falls below such standard;
- if it bears or contains any artificial flavoring, artificial coloring, or chemical preservative, unless it bears labeling stating that fact; or
- if it fails to bear, directly thereon or on its container, the inspection legend and other information required by the Secretary to help ensure proper handling by the consumer.

When USDA inspects meat or meat food products, it will apply what is known as the "official mark," the official inspection legend, or any other symbol prescribed by regulations of the Secretary; the "official inspection legend" is the symbol prescribed by regulations showing that an article was inspected and passed in accordance with the FMIA.

All proposed, interim final, and final rules governing the meat industry are published in the *Federal Register* by the Office of the Federal Register, National Archives and Records Administration. The *Code of Federal Regulations* (CFR) is the codification of the general and permanent rules published in the *Federal Register* by the executive departments and agencies of the US government. The CFR is divided into 50 titles that represent broad areas subject to federal regulation. Most of the rules governing the safe manufacturing of meat and poultry are found in Title 9, Animals and Animal Products, Chapter III, FSIS, Department of Agriculture.

The Regulatory Process

The current regulatory initiatives for meat hygiene in the US are based in large part on the pathogen reduction/hazard analysis critical control point (PR/HACCP) rule (FSIS, 1996a). The regulatory initiatives include extensive sampling and testing of the environment, raw materials, and finished products and additional inspection processes such as in-depth verifications and food safety assessments (FSAs) that are completed by FSIS inspection teams. These additional inspection activities address noncompliance issues, review validation data used to support critical control points, and include additional verification activities such as extensive sampling and testing of product and non-product contact surfaces, as well as finished products.

The PR/HACCP rule requires that all slaughter and processing plants adopt a HACCP system of process control to *prevent* food safety hazards. HACCP plans typically help to reduce and control hazards and in fewer instances, where there are kill steps, to eliminate hazards; but FSIS takes a regulatory approach that HACCP will prevent hazards from being present in the final product, currently an unrealistic concept for raw meat products. In the PR/HACCP

rule, FSIS stated that they wanted to *minimize* regulatory burdens on the industry and that the performance criteria would be implemented on the basis of a statistical evaluation of the prevalence of bacteria in each establishment's products measured against the nationwide prevalence of the bacteria in the same products. FSIS has not fulfilled this obligation in the last 12 years.

In the PR/HACCP rule, FSIS stated that they were working with industry, academia, and governmental agencies to *develop and foster* measures that can be taken on the farm and through distribution and marketing of animals to reduce food safety hazards associated with animals presented for slaughter. Twelve years have yet to deliver the interventions that can be used on the farm and throughout distribution and marketing of animals, although finally in February 2008 USDA granted a conditional license to Bioniche, a Canadian biopharmaceutical company, for its *Escherichia coli* O157:H7 cattle vaccine to collect data to move the product to full licensure. FSIS (1998a) stated that the PR/HACCP regulations "provide enormous flexibility for the industry to develop and implement innovative measures for producing safe foods." Despite the creation in 2003 of the Office of New Technology within FSIS, there have been examples of relatively straightforward interventions (e.g., higher levels of organic acids, hydronium ion formulations, chlorine dioxide, carcass irradiation) taking months, if not years, to move through the approval system that was supposed to expedite technology transfer; at this time, many of these interventions remain submissions without FSIS approval for commercial application.

FSIS (1996b) stated that the use of microbiological performance standards was part of a fundamental shift in FSIS regulatory philosophy and strategy, from command and control (telling how) to performance standards (express the objectives without specifying the means). As much as FSIS has spoken about changes to the inspection system, there is a lack of evidence that they have moved away from command-and-control inspection. Unionized inspection staff, by and large the same inspection staff that was in existence before the PR/HACCP rule, generally still operates under command and control, rather than a cooperative, educational process with the establishments producing meat products. FSIS also has taken it upon itself to develop best practices for meat hygiene, e.g., production of raw ground beef, without any cooperative involvement from industry or other stakeholders. These best practices become operating expectations, additional command-and-control initiatives instructing establishments "how" they must operate to comply with FSIS expectations.

After reviewing the FSIS regulatory approach to performance standards, a major limitation is the lack of involvement of all stakeholders in the process before performance standards are published. There is a post-publication comment period, but better performance standards could be developed if the process was transparent and open to all stakeholders earlier, with full public disclosure and debate. Through such a process, all data and data gaps, social and political concerns, risk assessment and risk management issues, and risk-benefit analyses could be debated. The compromises would be gained through

consensus building such that the final performance standard or criteria would have a greater likelihood of being embraced, or at least understood, by all stakeholders at the time of publication.

FSIS (2006) published guidance (e.g., FSIS Directive 5000.1) for their inspection staff on how they are to protect public health by properly verifying an establishment's compliance with the pathogen reduction, sanitation, and HACCP regulations. These procedures are prescriptive and are used in verifying sanitation performance standards in 9 CFR 416 involving grounds and pest control, construction, lighting, ventilation, plumbing, sewage disposal, water supply and water, ice, and solution reuse requirements, dressing rooms and lavatories, equipment and utensils, sanitary operations, and employee hygiene. FSIS has not defined the science behind these regulations in sufficient detail to clarify when and how specific violations can lead to regulatory action and more importantly to actual food safety risks.

FSIS inspection personnel are only required to be of the opinion that conditions *may have* caused product to be contaminated with filth or cause product to be unsafe. FSIS gives their inspectors the right to use professional knowledge and judgment in making the determination whether the sanitation performance standard requirements are met. However, there is no visible or transparent process in place to measure the abilities of inspection staff to correctly and consistently make such judgments. FSIS publishes quarterly enforcement data that include the number of FSIS HACCP checks performed, the percentage of these checks that resulted in noncompliance records (NRs), the number of these NRs that were appealed, and the number of appeals that were granted or modified. In theory, as inspectors become better educated and trained, the number of appeals should decrease as establishments recognize that the inspectors correctly identify noncompliance issues; and the number of appeals granted and modified (indicating that the inspector did not accurately assess the NR issue) should decrease as well. An analysis of quarterly enforcement data from 2003 through 2007 has indicated that the number of NRs appealed remained fairly constant at 1.5%, the number of appeals granted remained between 30 and 40%, and the number of NRs modified remained near 15%. These data illustrate that FSIS inspection staff continue to incorrectly assess NR issues; this is wasteful because the appeal process is cumbersome, time- and resource-consuming, and often not undertaken because of these characteristics and for fear of repercussions by local inspectors being challenged through the appeal process. FSIS has not shared publicly measurement data, if they are collected and analyzed, to determine the root causes of incorrect assessments by FSIS inspectors. When questioned in FSIS industry meetings about the quarterly enforcement data, FSIS appeared indifferent to the data and, to date, have not shared any strategies to improve inspector performance relative to the issuance of NRs.

The regulatory process includes a review of validation data used to support CCPs and HACCP plan decision-making at USDA-inspected establishments.

Complicating factors surrounding validation include the variation in acceptance by local regulatory authorities of supporting documentation, e.g., inconsistency in the acceptance of published literature as satisfactory validation documentation. A regulatory authority needs only to question the legitimacy of the validation documentation, without providing a rationale for its questioning or without providing an expectation for what is required to address its question. That is, the USDA-inspected establishment can be left guessing as to what is required to satisfy a local authority and have no guarantee that the validation data, even if scientifically sound, will prevail in satisfying a regulatory authority. As a result, acceptance of validation data is somewhat arbitrary, as regulatory authorities have not established, in most cases, specific criteria for acceptable validation documentation. Industry contends that until such criteria are established, or a set of validation documents is recognized for specific CCPs, the ambiguities and inconsistencies will persist as challenges for industry and FSIS.

The regulatory process has as its basis, rules (laws) that are developed and implemented according to a legal process of review, economic impact analysis, public comment, and governmental oversight. These checks and balances ensure that stakeholders have the opportunity to participate, if only through comments and lobbying efforts. The process takes time and is an administrative burden to FSIS as it attempts to affect its regulatory policies. To circumvent this process, FSIS uses directives and notices, which are defined to be directions to its field inspection staff, and *Federal Register* notices to essentially expand its regulatory authority and require changes to industry practices. FSIS has justified this approach by stating that such directives and notices are simply interpretations of the PR/HACCP rule and do not represent attempts to issue new policy. Directives and notices can easily number 100 annually, whereas rules generally number less than five. FSIS uses directives and notices to instruct its staff what to do in meat slaughter and processing establishments; these new inspection practices by their nature translate into new requirements for industry. These instruments effectively have mandated particular testing regimes, outcomes of hazard analyses, labeling requirements, and other expectations developed in the absence of stakeholder input that is critical to rule making. The regulation of meat processing establishments would be different than it is in 2008 if FSIS was required to use rule making to establish all regulatory policies.

Since 2005, FSIS has promoted its position as a public health agency, even though the FMIA did not specifically indicate this intention; most persons consider the US Centers for Disease Control and Prevention (CDC) as the US public health agency. FSIS acknowledges, but has done little to overcome the limitations of the attribution data available to link human illnesses to specific meat products; however, even without such measurement data, FSIS continues to build its regulatory base of authority in hopes of reducing human disease.

Regulatory Enforcement Actions

The rules of practice are described in 9 CFR 500 and define regulatory control, withholding actions, and suspension actions that can be taken by FSIS. Regulatory control actions are defined as the retention of product, rejection of equipment or facilities, slowing or stopping of lines, or refusal to allow the processing of specifically identified products. A withholding action is the refusal to allow the marks of inspection to be applied to all products or specific products produced by an establishment. A suspension is an interruption in the assignment of a FSIS inspector to all or part of an establishment, thereby halting operations.

Regulatory control actions may be warranted for direct product contamination with a contaminant that does not result in a food safety hazard, for product that is economically adulterated, and for regulatory noncompliance even when there is no product contamination or adulteration. For example, a regulatory control action would be taken when inspection program personnel are assessing sanitary conditions of the establishment before operation and observe product residue from the previous day's production on a contact surface, or if inspection program personnel determine that packaged product does not meet the net weight requirements. Inspection program personnel could initiate a regulatory control action when there is noncompliance with the sanitation performance standard regulations, if control is needed to prevent contamination of product. Regulatory control actions are not frequently used for HACCP regulatory noncompliance unless control is necessary to prevent shipment of contaminated or adulterated product. As part of a regulatory control action, FSIS inspectors will issue a NR. There will be a trend analysis to determine if the establishment has repetitive NRs for similar problems, suggesting that the preventive actions have not been successful in preventing recurrence of a noncompliance issue. Linking these repetitive NRs is one means that is used by FSIS to accelerate and implement additional inspection and control actions at individual establishments.

Withholding actions may be taken if the establishment produced and shipped adulterated or misbranded product, does not have a HACCP plan, does not have sanitation standard operating procedures (SSOPs), or is operating with sanitary conditions that could lead to adulteration. Withholding actions also can be taken if the establishment violated the terms of a regulatory control action; had an operator, officer, employee, or agent that assaulted, threatened to assault, intimidated, or interfered with a FSIS employee; did not destroy a condemned meat or poultry carcass, or part or product thereof, within three days of notification; or handled or slaughtered animals inhumanely. Withholding or suspension actions also can be taken if the HACCP plan is found to be inadequate, SSOPs are not implemented or maintained, *Salmonella* performance standards have not been met, or violations of other rules are detected or suspected.

When a withholding or suspension occurs, the establishment may or may not be notified in advance of the action, depending on whether or not there is an imminent threat to public health. A notice of intended enforcement (NOIE) is issued to provide notification to an establishment that there is a basis for FSIS to withhold the mark of inspection or to suspend inspection. FSIS issues an NOIE to an establishment for noncompliances that do not pose an imminent threat to public health but that may warrant the withholding of the mark of inspection or suspension of inspection if not corrected. In addition to informing an establishment about noncompliances warranting a withholding or suspension, the NOIE provides an establishment three business days to contest the basis for the proposed enforcement action or to demonstrate how compliance has been or will be achieved.

FSIS assesses and evaluates the establishment's response to the NOIE and decides whether inspection should be withheld or suspended. FSIS determines whether the establishment's proposed action plan addresses the problem and, if implemented, is likely to provide an acceptable solution. FSIS will consider the establishment's history of implementing its operating procedures and its planned corrective and preventive actions and determine whether the establishment is likely to implement its proposed actions effectively. FSIS may hold a suspension in abeyance and allow the establishment to operate under the conditions agreed to by FSIS if the establishment presents a plan that demonstrates to the satisfaction of FSIS that the establishment has designed corrective and preventive actions that are appropriate to meet the regulatory requirement and ensure that it will not recur. The suspension in abeyance may be used to allow the establishment to operate after implementing these corrective and preventive actions, so FSIS can determine whether the establishment is able to adequately execute the plan. If FSIS decides to put the suspension in abeyance, and the establishment fails to either meet regulatory requirements or maintain regulatory compliance during the abeyance period, FSIS may lift the abeyance and put the suspension back in effect. If this occurs, FSIS will suspend inspection and immediately notify the establishment management. Again, the establishment has three business days from receipt of the written notification to respond to FSIS unless the time period is extended by FSIS. The establishment may make immediate corrective actions and further plan preventive actions or appeal the withholding or suspension action. FSIS also can file a complaint to withdraw a grant of federal (USDA) inspection for the reasons specified for withholding or suspension actions.

One of the problems with FSIS regulatory enforcement actions is that there are no measures of consistent issuance of NRs by the thousands of FSIS inspectors, and thus, inequities in regulatory enforcement actions very likely occur in USDA-inspected establishments. Even more troubling is the use of NOIEs. If an establishment has been operating for months or years without a NOIE and is operating during a FSA, for example, when the HACCP plan and other operating parameters are under scrutiny, and product is being produced and shipped with the USDA mark of inspection, it is difficult to understand

how FSIS can conclude that unless some aspect of the HACCP plan or other operating parameters are changed, the plant is subject to withholding or suspension actions within a three-day time period. There is no rationale explanation provided as to how products can be produced for weeks or months with the mark of inspection applied and then suddenly, overnight, those same products represent a risk to the consumers or other customers. FSIS has not provided such a rationale and continues to use NOIEs to force changes that may or may not add any additional food safety protection. Establishments producing meat products are often forced to propose and implement changes to food safety systems when there is no scientific justification for such changes. For example, a fabrication establishment that buys carcasses and converts them to meat cuts and trimmings can be required by regulatory authorities to include *E. coli* O157:H7 as a hazard reasonably likely to occur in its HACCP plan, without measurable criteria to define "reasonably likely to occur," forcing them to have a CCP to control the hazard. Yet, in such operations there are no CCPs available to control the hazard. Interventions such as organic acid sprays are not effective enough to be considered a CCP. Often such establishments have no alternative but to use microbiological testing as a regulatory CCP. Thus, FSIS uses regulatory enforcement actions to force regulatory HACCP to change science-based HACCP plans.

Development and Use of Performance Standards by FSIS

The PR/HACCP rule established the use of microbiological performance standards as key components of the regulatory process for meat hygiene. Under the regulations in 9 CFR 417, critical limits must be designed to satisfy relevant FSIS regulations, including performance standards. FSIS considers developing HACCP systems around verifiable, objective performance standards as the most effective way for establishments to consistently produce safe, unadulterated meat and poultry products. However, it is questionable whether FSIS understands that while microbiological performance standards are valuable and useful tools to define an expected level of control in one or more steps in the process and to achieve public health goals to reduce the number of foodborne illnesses, performance standards must be based on a set of principles including the relationship of the standard to the public health goals. The stringency of a performance standard should be proportional to the risk and stated public health goals and should consider the degrees of uncertainty.

The continued use, or revision of existing performance standards, and establishment of new performance standards by FSIS without following principles established by experts such as those in the International Commission on Microbiological Specifications for Foods (ICMSF) and the US National Advisory Committee on Microbiological Criteria for Foods (NACMCF) create unjustified and arbitrary regulation of the meat slaughter and processing industries.

As early as 2003, a National Academy of Sciences (NAS) committee (2003) concluded that improvements are needed in the design of FSIS performance standards, in particular, that FSIS needs to bring regulatory HACCP in line with science-based HACCP. The ICMSF, NACMCF, and NAS reported that the use of single-value, worst-case estimates as a means of considering uncertainty should be avoided, particularly when more than one factor contributes to overall public health risk. FSIS has not provided the scientific rationale for their worst-case estimates for use in the design of performance standards. FSIS has stated historically that assumptions are "conservative but reasonable," yet simultaneously that worst-case levels are not expected to actually occur (FSIS, 2001).

When designing microbiological standards as performance standards, the principles for the establishment of microbiological criteria developed by the Codex Alimentarius Commission (Codex, 1997) should be followed. These principles state that a microbiological criterion should be used only where there is a definite need. Application of the standard should be practical and technically attainable by applying good hygiene practices (GHP) and HACCP. The standard should accomplish the intended purpose, e.g., reducing foodborne illnesses. These Codex principles developed for microbiological criteria have not been applied consistently in the design of performance standards in the US, particularly as they apply to meat and poultry products.

International harmonization of performance criteria, including performance standards, and the bases on which they are set, is important scientifically as well as for trade. Internationally, performance criteria are the effects in frequency and concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a performance objective or a food safety objective (FSO). A performance objective refers to the maximum frequency and concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to a FSO or acceptable level of protection. A FSO, based on a tolerable level of risk, helps to establish the performance of a food process that would ensure that, at the moment of consumption, the level of the hazard in a food would not be greater than the FSO. The NAS committee (2003) recommended at least five years ago that FSIS adopt the concept of a FSO. ICMSF (2002) recognized that since GHP and HACCP are the primary tools available to help industry control microbiological hazards in food operations, it is essential that the technical achievability of the FSO be confirmed. FSIS regulations on meat hygiene do not reflect the use of a FSO, and currently, there appears to be no attempt to design regulations around FSOs despite the long-standing recommendations from the international and US scientific communities.

One of the most important factors in establishing performance standards for meat is to be able to measure the impact of the performance standards on public health. Without specific product handling–illness linkages, it is nearly impossible to determine whether a performance standard truly is reducing foodborne disease related to a food product. Six years ago, NACMCF (2002) concluded

that existing public health statistics for meat products make it very difficult to specifically attribute reductions in enteric diseases to the performance standards enforced by FSIS. Most recently, the Center for Science in the Public Interest (CSPI, 2007b) reviewed the CDC data and concluded that the majority of reported foodborne outbreaks do not have complete outbreak information; over half of all outbreaks reported to CDC had no etiology or food vehicle. CSPI recommended that CDC should improve outbreak reporting and surveillance and lead all states to follow national standards for tracking disease outbreaks.

CDC's Emerging Infections Program, in cooperation with USDA, the Food and Drug Administration (FDA), and at least 10 states, uses the foodborne diseases active surveillance network (FoodNet) to determine the burden of foodborne disease in the US, to monitor trends in the burden of specific foodborne illness over time, to attribute the burden of foodborne illness to specific foods and settings (an objective not yet met), and to develop and assess interventions to reduce the burden of foodborne disease. CDC also coordinates PulseNet, a national network of over 75 public health and regulatory laboratories designed to detect clusters of foodborne disease cases by pulse field gel electrophoresis (PFGE). The PFGE patterns are submitted electronically to a database that is available on demand to registered participants for rapid comparison of the results; the number of PFGE patterns submitted to PulseNet databases has increased from less than 10,000 in 1998 to over 60,000 in 2007. PulseNet is designed to allow for real-time communication among state, local, and international laboratories; to facilitate early identification of common source outbreaks; and to help FDA and USDA identify areas where implementation of new measures are likely to increase the safety of the food supply. CDC also uses its foodborne disease outbreak response and surveillance team to conduct national surveillance on foodborne infections and outbreaks of foodborne illness and to assist in the investigation of foodborne disease outbreaks. With all of these activities, progress on developing useful attribution data remains slow and limited; improving attribution data has not been a top priority for regulatory authorities despite the need expressed from a broad stakeholder base.

Salmonella Performance Standards

The PR/HACCP rule sets performance standards for establishments slaughtering selected classes of food animals or producing selected classes of raw ground products to verify that industry systems are effective in controlling the contamination of raw meat products with bacterial pathogens. Raw products with specified performance standards include carcasses of cows and bulls, steers and heifers, market hogs, turkeys, and broilers. Processed raw products assessed against performance standards include ground beef, ground chicken, and

ground turkey. The *Salmonella* performance standards for these product classes are based on the prevalence of *Salmonella* as determined from FSIS' nationwide microbiological baseline studies. FSIS inspection personnel verify that establishments are meeting the standards by collecting and testing targeted and randomly selected product samples.

Before 2006, FSIS collected non-targeted or "A" set samples at establishments randomly selected from the population of eligible establishments, with a goal of scheduling every eligible establishment at least once a year. Other codes (such as B, C, and D) represented sample sets collected from establishments targeted for follow-up testing following a failed "A" set. Beginning June 2006, establishments were scheduled based on new risk-based criteria to focus FSIS resources on establishments with the most samples positive for *Salmonella* and the greatest number of samples with serotypes most frequently associated with human salmonellosis, as identified by CDC. In 2008, FSIS began publishing *Salmonella*-positive rates associated with specific USDA-inspected establishments, categorized by their performance against the existing *Salmonella* performance standards, apparently in an attempt to use pressure by commercial customers and consumers to drive down *Salmonella*-positive rates. The categories are misleading since to be in Category 1 an establishment must be below 50% of the existing performance standard; to be in Category 2 means that the establishment is meeting the performance standard, but above the 50% value; the remainder of establishments are in Category 3. Many retailers will only want to purchase from Category 1 establishments, even though Category 2 establishments meet the performance standard; and Category 3 establishments only ship products into commerce that meet USDA requirements, including the *Salmonella* performance standards.

Isolates from *Salmonella*-positive samples are serotyped at the USDA Animal and Plant Health Inspection Service's National Veterinary Services Laboratory (APHIS NVSL). *Salmonella* testing and serotype data, along with complementary data from molecular and phenotypic analyses, provide an opportunity to examine the association among serotypes isolated on-farm, from meat and poultry products, and from human cases of salmonellosis, i.e., an attempt at attribution. Unfortunately, to date, the cooperative effort between FSIS, APHIS NVSL, CDC, and other state and federal governmental agencies has not optimized the use of genetic typing data to describe foodborne disease as it relates to the entire US food supply.

Some of the more common serotypes isolated from meat and poultry products are rarely isolated from human patients. Conversely, some of the serotypes frequently found in human cases of salmonellosis are found in various meat and poultry products. Serotypes identified from human cases of salmonellosis can also be found in other food and non-food sources. CDC has identified Typhimurium, Enteritidis, Newport, Javiana, Montevideo, Heidelberg, and I 4,[5],12:i:- as seven commonly identified serotypes causing human infections in the USA. Combined, these serotypes accounted for a majority (64%) of human infections in the US according to the 2006 FoodNet data.

Kentucky, Hadar, and Derby, the predominant serotypes identified in 2006 from meat and poultry products, were not found among the top seven serotypes identified in human surveillance data.

FSIS designed the *Salmonella* performance standards to verify the adequacy of HACCP systems or, in other words, to verify process control in slaughter and ground meat operations. Industry questioned whether FSIS had the statutory authority to take the enforcement actions laid out in the policy. On November 30, 1999, a Texas meat processor making raw ground beef filed suit challenging a suspension of inspection by FSIS for failing to meet the *Salmonella* performance standard. On May 25, 2000, a decision was rendered in Supreme Beef Processors, Inc. vs. USDA (Civil Action No. 3:99-CV-2713-G) in favor of the company. A US district court ruled that FSIS lacked the statutory authority to suspend inspection due to the establishment's failure to comply with the *Salmonella* performance standard. In December 2001, the US Court of Appeals for the Fifth Circuit ruled that the *Salmonella* performance standard conflicts with the statutory language in the FMIA and therefore is invalid. The appellate court also rejected USDA's argument that the *Salmonella* performance standard should be upheld because it serves as a measure of whether pathogens that are adulterants, such as *E. coli* O157:H7, are also present in products. The court stated that because the performance standard measures *Salmonella* in the final product but not in the incoming raw materials, it cannot "serve as a proxy for cross contamination because there is no determination of the incoming *Salmonella* baseline," a position later endorsed by the NAS committee (2003). Despite these historical court opinions, FSIS continues to use and enforce *Salmonella* performance standards for a multitude of products, but thus far has avoided making judgments regarding the sanitary aspects of the processing establishments.

Sperber (2005) provides an excellent discussion of the PR/HACCP rule and the disconnect between the enactment and the enforcement of the *Salmonella* performance standard and any direct food safety or public health protection benefit. Sperber described performance standards as "the black holes of food safety – all of the light is sucked out of a system and it never returns," and concluded that the "*Salmonella* performance standard is perhaps the most opaque and unfortunate flaw in the Megareg [PR/HACCP rule]."

Zero-Tolerance Standard for *E. coli* O157:H7

E. coli O157:H7 has been a concern in the US meat industry since the 1993 outbreak in the US Pacific Northwest and since FSIS declared the pathogen to be an adulterant in raw ground beef in 1994. In January 1999, FSIS expanded the zero-tolerance policy such that beef products that have been injected or mechanically tenderized are considered adulterated if *E. coli* O157:H7 is found and the products are not processed into RTE items. In addition, intact

cuts that are to be processed into non-intact products (e.g., raw ground beef) before distribution are considered adulterated if *E. coli* O157:H7 is found.

The PR/HACCP rule requires USDA-inspected establishments to implement controls over critical operations to reduce risks from hazards deemed likely to occur through the hazard analysis process; in 2002 a FSIS notice stated that at any level whatsoever, *E. coli* O157:H7 is a hazard reasonably likely to occur. This exemplifies the problem that although FSIS expects processors to determine the likelihood of a hazard to occur, FSIS also will dictate, based on their assumptions and beliefs, when and where it believes a hazard is reasonably likely to occur regardless of a processor's hazard analysis. According to FSIS, this is the case for *E. coli* O157:H7 in beef raw materials destined for raw, comminuted beef regardless of the prevalence of the organism in the raw materials. Additionally, a subsequent publication by FSIS in May 2005 made it clear that "clean-up to clean-up" is no longer an adequate basis for lot definition if testing of the raw materials is not conducted in a manner that supports a distinction between raw materials used on separate days.

The ramifications of positive test results are significant; every time there is a positive finding of *E. coli* O157:H7, it can trigger recalls, HACCP reassessment activities, and intensified inspection, sampling, and testing by FSIS at the manufacturing establishment and the suppliers of the raw materials to that establishment. The positive finding of *E. coli* O157:H7 also generates media inquiries on the perceived failures of the FSIS and industry control programs, legislative pressures to improve compliance with the zero-tolerance standard, and consumer activists' demands for additional regulatory and enforcement actions.

Because of a slight increase in the number of positive results and outbreaks in 2007, FSIS undertook a series of actions in an attempt to reduce the likelihood that raw, non-intact products will be adulterated with *E. coli* O157:H7. FSIS increased the number of verification samples throughout the supply chain from slaughter to the production of raw, ground beef and other raw, non-intact products such as needle-tenderized and enhanced products. FSIS notices in 2007 notified inspection staff to conduct follow-up sampling at slaughter establishments that supplied the carcasses that were fabricated to produce the raw materials used in the production of raw ground beef products that tested positive for *E. coli* O157:H7 and notified inspection staff to implement an increased sampling ($n = 60$) and testing program for beef trimmings, including trimmings from sub-primal cuts, that are destined for raw, non-intact beef products. In October 2007, additional notices were issued instructing FSIS district offices to schedule FSAs at establishments within 30 days of a positive test result for *E. coli* O157:H7 in a raw beef product; to implement a stepped-up sampling and testing program for raw ground beef, raw ground beef trimmings, and other ground beef raw materials in response to a positive test result for *E. coli* O157:H7; and to require the implicated establishment to reassess its

HACCP plan, justify their use of interventions and controls for beef slaughter and fabrication, and document changes made in response to the reassessment.

In 2007, FSIS required the inspection staff to complete a survey in regard to how establishments that process raw beef address and control *E. coli* O157:H7. According to FSIS, the results of this survey would be used to proclaim best practices or minimal expectations for beef slaughter and processing establishments. FSIS reported that this will enable them to move toward a more risk-based inspection (RBI) process overseeing the production of raw, non-intact beef products by focusing on establishments with the fewest implemented best practices. In effect, these best practices become regulatory requirements.

The zero-tolerance standard for *E. coli* O157:H7 also impacts international trade. Because of apparent lapses in operational and testing controls for *E. coli* O157:H7 at a single Canadian supplier exporting to the US in 2007, FSIS and the USDA Office of International Affairs required all beef processors that export raw beef to the US to re-evaluate and resubmit their *E. coli* O157:H7 control programs for approval as equivalent, regardless of the fact that *E. coli* O157:H7 may not be the shiga toxin-producing *E. coli* of primary concern as a potential contaminant and human pathogen in the exporting country. The key components of these revised programs were the sampling and testing frequencies and the definition of lot size based on the verification testing program. Despite repeated requests from industry for labeling of imported trimmings that are destined for cook operations (and thus outside of the focus on the control of *E. coli* O157:H7 destined for raw, non-intact products), FSIS decided not to allow any labeling of imported raw beef that would separate product destined for cooking from that destined for use in raw, non-intact products and thus failed to optimize RBI for sampling and testing of imported trimmings.

USDA- and state-inspected establishments are required to establish control measures that result in processes and products that meet performance standards established by regulatory authorities, regardless of whether the performance standards are achievable with existing technologies (e.g., zero tolerance for *E. coli* O157:H7 in ground beef raw materials). This creates situations where the science clearly establishes the inability to be in compliance, yet regulatory HACCP demands "artificial compliance," that is, where control measures reduce levels as low as possible, or below detectable levels, even though clearly a zero tolerance is unachievable today using existing, approved interventions (except irradiation which has limited approval and is generally not accepted and preferred by the US consumer). Thus, the challenge becomes one of validating that a control measure achieves an unattainable goal. Clearly, this is not an approach that any scientist wishes to undertake; but often the regulatory approach to HACCP leaves USDA-inspected establishments with no other option.

FSIS has not yet recognized the fallacy of a zero-tolerance standard for *E. coli* O157:H7 and apparently believes that the zero-tolerance standard, in the absence of accepted technology to achieve this standard, and in combination with sampling, testing, and the diversion of millions of kilograms of raw

beef, is scientifically and morally justified. In fact, in 2008 FSIS began discussions surrounding the expansion of the zero-tolerance standard to include other shiga toxin-producing *E. coli*; this would have significant ramifications for the meat industry in the US and exporting countries. The zero-tolerance policy was popular with consumers, media, and legislative representatives; thus, it apparently was deemed adequately designed and developed according to FSIS. The essential element for consumer protection remains appropriate handling and cooking, as unpopular as it is to suggest that consumers execute their responsibilities.

Stabilization/Cooling Performance Standards

Stabilization/cooling performance standards for preventing the growth of spore-forming bacteria are given in 9 CFR 318 for RTE roast beef, cooked beef, and corned beef products; fully cooked, partially cooked, and char-marked meat patties; and certain partially cooked and RTE poultry products. The regulations and supporting documents specify two performance criteria for chilling: (1) there can be no multiplication of toxigenic microorganisms such as *Clostridium botulinum*, and no more than a 10-fold multiplication of *C. perfringens*, within the product and (2) *C. perfringens* shall not exceed 100,000/g after chilling.

Data from FSIS microbiological surveys led to the use of a "worst-case" scenario based on 10,000 *C. perfringens* per gram of raw product to develop the performance standard, even though FSIS data showed that only a very small percentage of samples had concentrations exceeding 1,000 organisms per gram and only one sample had an estimated concentration of more than 10,000 cells per gram. Industry data have shown that the prevalence of *C. perfringens* in raw meat and poultry is very low, generally 0 to <100/g (Buege & Ingham, 2003; Greenberg, Tompkin, Bladel, Kittaka, & Anellis, 1966; Taorima, Bartholomew, & Dorsa, 2003). An extensive summary of industry data for products tested following cooling deviations showed the prevalence and concentration of *C. perfringens* in cooked products were very low, even in products that failed to meet the chilling requirements (Kalinowski, Bodnaruk, & Tompkin, 2001). Thus, the statistical estimates and rationale provided by FSIS (FSIS, 1998) to consider *C. perfringens* a hazard reasonably likely to occur and to require a restrictive cooling performance standard for a wide range of products are not supported by data.

With the possible exception of certain heavily spiced foods that have non-inhibitory pH values, water activities, nitrite concentrations, or salt levels, the microbiological hazards, *C. perfringens* and *C. botulinum*, would not be hazards reasonably likely to occur during chilling of meat products at USDA-inspected processing establishments. Historical reviews of the literature (Bean & Griffin, 1990; Bret & Gilbert, 1997; CSPI, 2000; Hobbs, 1979; Tompkin, 1983) and

currently available food attribution data indicate that no reported outbreaks have occurred from improper chilling of a cooked meat product in a USDA-inspected processing establishment; root causes of outbreaks related to *C. perfringens* primarily are improper holding temperatures in restaurants, cafeterias, catering operations, and delis.

Despite the lack of evidence that the original May 1988 guidance on chilling (FSIS Directive 7110.3) resulted in products that presented a risk to public health, FSIS tightened the chilling requirements. Even with the more restrictive requirements issued in June 1999, FSIS went on to state that there was little margin for safety with the new required chilling times and temperatures. FSIS never clarified their rationale for the more restrictive performance standards; and these performance standards have not improved public health in a measurable way.

Similar conclusions to those reached for *C. perfringens* can be reached for *C. botulinum*, but the severity of botulism warrants further consideration. In contrast to *C. perfringens*, there have been outbreaks of botulism throughout the world involving both cured and non-cured meats. However, an examination of currently available food attribution data and historical reviews (Tompkin, 1980) indicate that there have been no incidents of botulism in the US due to inadequate chilling of cooked perishable meat products produced under USDA or state inspection.

Zero-Tolerance Standard for *Listeria monocytogenes* in RTE Foods

From 1985 until 2008, FDA maintained a policy of zero tolerance for *L. monocytogenes* in RTE foods that may be consumed without further preparation by the consumer. FDA considers RTE foods to be adulterated under the Federal Food, Drug and Cosmetic Act (FFDCA, 21 USC 301 *et. seq.*) if any *L. monocytogenes* is detected in either of two 25-g samples (i.e., 0.04 colony forming units per gram). Since 1989, FSIS has maintained a similar zero-tolerance policy for RTE meat products. Meat products in RTE form in which any *L. monocytogenes* is detected are deemed adulterated under the FMIA. The regulatory status of non-RTE products that contain *L. monocytogenes* is determined on a case-by-case basis, but such products may be subject to public health alerts or voluntary recalls as well, e.g., if cooking instructions are judged by FSIS to be inadequate to ensure safety.

A substantial body of evidence now demonstrates that the zero-tolerance policy for *L. monocytogenes* is scientifically unsupportable, especially when applied to foods that do not support the growth of *L. monocytogenes*. Twenty years ago, the WHO (1988) concluded, "The total elimination of *L. monocytogenes* from all food is impractical and may be impossible." Over six years ago, ICMSF (2002) advised, "due to its widespread prevalence in the environment, eradication of *L. monocytogenes* from the food supply is impossible."

Internationally, Canada, the United Kingdom, Australia, and New Zealand have established that zero tolerance is not an appropriate regulatory strategy for *L. monocytogenes* in all circumstances for all foods. The European Food Safety Authority Scientific Panel on Biological Hazards also endorses a categorical approach with either an absence in 25 g or <100 colony forming units per gram at the time of consumption being appropriate criteria depending upon the category of food, e.g., foods intended for sensitive populations or foods not supporting growth of *L. monocytogenes*, respectively. It was established early in the risk assessment process that a FSO of <100 *L. monocytogenes* per gram in products that do not support growth provides a higher level of protection than does a more strict tolerance of "not detected in 25 g" (ICMSF, 2002; Todd, 2002; Lund, 2000; Ross, Todd, & Smith, 2000).

The food industry has engaged in unprecedented efforts to eradicate *L. monocytogenes* from the processing environment. Despite continuing success, elimination of *L. monocytogenes* remains a constant challenge because the organism is ubiquitous in many of the raw materials entering the plant every day and resilient and persistent in the refrigerated processing environments. AMI, along with 14 other food trade associations, formed the Alliance for Listeriosis Prevention (the Alliance). The Alliance studied the science and the legal and regulatory issues surrounding the zero-tolerance standard. The result of these deliberations was the development of a science-based position that certain RTE foods present a substantially lower risk from *L. monocytogenes* and therefore should not be subject to the zero-tolerance standard. In December 2003 the Alliance submitted a petition requesting FDA consider establishing a regulatory limit of 100 *L. monocytogenes* per gram in RTE foods that do not support the growth of the bacterium (Alliance for Listeriosis Prevention, 2003). The proposal is based on evidence that consumer protection is a function of cell number and not the mere presence of *L. monocytogenes*. A quantitative risk assessment based on an extensive survey of *L. monocytogenes* in RTE foods predicted that elimination of high concentrations of *L. monocytogenes* in such foods could reduce listeriosis as much as 99.5% (Chen, Ross, Scott, & Gombas, 2003; Gombas, Chen, Clavero, & Scott, 2003). The FDA/FSIS risk assessment for *L. monocytogenes* concluded "exposures to *L. monocytogenes* seldom lead to listeriosis, even among highly susceptible segments of the population" (FDA & FSIS, 2003).

In May 2005, the Alliance submitted a citizen's petition to FSIS requesting a regulatory limit for *L. monocytogenes* for RTE meat products that do not support the growth of *L. monocytogenes*. Four months later, the Alliance received notification from FSIS that the citizen's petition had been denied, without prejudice to its revision and resubmission at a later date. In the response to the Alliance, FSIS stated that they "think the concept of a quantitative pathogen limit for certain products under carefully defined circumstances may have merit"; however, they cited several regulatory and scientific concerns related to such a policy. Some of these concerns included the definition of *L. monocytogenes* growth, the appropriate statistical sampling schemes, the

quantitative methodologies, potential for cross-contamination at retail and the home, and the human infectious dose for *L. monocytogenes*. In December 2006 the Alliance submitted a new petition to FSIS addressing the questions raised in the first denial letter with additional scientific support. FSIS has shown no interest in proactively addressing these petitions for change nor has FSIS used the scientific data to refine their regulatory policies on *L. monocytogenes* in RTE meat products. However, in February 2008 FDA announced its intention to consider the presence of low levels ($\leq$100 colony forming units per gram) of *L. monocytogenes* in foods that do not allow growth of *L. monocytogenes* throughout the life of the product as not a violation of their *L. monocytogenes* policy. This was a significant change for FDA; it remains to be seen whether actual regulatory changes will occur and whether FSIS will alter their policy as well.

L. monocytogenes at Retail Delis

Much has been learned over the past 15 years regarding the control of *L. monocytogenes* in the meat processing environment of USDA-inspected establishments. Information on antimicrobial interventions to help reduce the potential for product contamination with *L. monocytogenes* has been widely distributed through AMI Foundation (AMIF) *Listeria* Intervention and Control workshops. However, the control of *Listeria* in retail deli environments has been questioned by government, trade associations, academicians, and consumer groups; and the prevalence of *Listeria* in products handled, sliced, and prepared in this environment can be higher than in case-ready products produced at the processing plant and sold intact through the retail establishments.

Control of *L. monocytogenes* in RTE meat products in USDA-inspected establishments is achieved through the application of a validated lethality step (e.g., cooking) combined with an aggressive environmental control and sanitation program that prevents the establishment of *L. monocytogenes* growth in a niche within the cooked products area of the plant. This control program must be continually verified with an aggressive environmental sampling and testing program. When products are destined to be further prepared or sliced in a retail customer deli, the product will have the opportunity to be re-exposed to a post-lethality processing environment that could inadvertently be contaminated with *L. monocytogenes*.

Surveys have provided insight into how frequently RTE meat products sliced in retail delis test positive for *L. monocytogenes*. Gombas et al. (2003) reported that a large survey of deli-prepared products indicated that the prevalence of *L. monocytogenes* in deli meats was 0.89% overall. When the samples were separated into those sliced at manufacturing compared to those sliced at delis, the prevalence was 0.4 and 2.7%, respectively. These data indicate that the deli products are increasing the exposure of consumers to *L. monocytogenes*.

A similar but larger study (n = 8000) presented at the 2006 International Association of Food Protection meeting found that the pre-packaged deli products had a prevalence rate of 0.15% while the deli-prepared products rate was 1.23% (Draughon, Ryser, Oyarzabal, Hajmeer, & Cliver, 2006). While these latter data may indicate an improving trend, it remains that the retail deli is a potential area for concern with respect to *Listeria* control. Surprisingly and with no explanation, FSIS expends almost no resources to better understand the risks at delis, but prefers to focus on the processing establishment. FSIS speaks of risk-based approaches to reducing public foodborne disease, yet, as evidenced by their approach to reducing exposure of consumers to *L. monocytogenes*, stops short of making a commitment to understanding root causes contributing significantly to foodborne disease throughout the supply chain.

Verification Sampling and Testing

A key component of the US regulatory scheme to ensure meat hygiene is FSIS verification sampling and testing conducted at USDA-inspected establishments. For example, in 1994 FSIS began a microbiological testing program to detect *E. coli* O157:H7 in raw ground beef. The objective of the testing program was to detect *E. coli* O157:H7 and to stimulate industry action to reduce the presence of the pathogen in raw ground beef. Since the initiation of the FSIS testing program, many grinders and suppliers of ground beef raw materials instituted slaughter interventions and routine sampling and testing programs for ground beef products and raw materials used in ground beef products. Over time, FSIS microbiological sampling and testing methods were modified at least three times to detect lower numbers of this pathogen through increased sample size and adoption of new more sensitive methods; however, FSIS did not adjust its targeted national prevalence to account for the improved methods, thereby creating a lower target without public disclosure.

Inasmuch as verification sampling and testing at USDA-inspected establishments comprise a regulatory program intended to assess the ability of meat slaughter and processing establishments to comply with existing, product-specific performance standards, the profiles of pathogen serotypes associated with selected raw materials and finished products, when detected, are not intended to indicate a national prevalence for a specific serotype within a respective product class. Despite limitations to interpretation, FSIS has used verification testing results as an indication of relative serotype distributions in raw products since implementation of the PR/HACCP rule.

Verification data developed by FSIS serve as a general measure of trends for industry compliance with performance standards. For example, in 2005 and 2006, 94.7 and 94.5%, respectively, of targeted sample sets of market hogs met the *Salmonella* performance standard; 3.7 and 4% of 6,648 and 7,242 samples,

respectively, were positive for *Salmonella*. In 2005 and 2006, 98.5 and 97.6%, respectively, of targeted sample sets of raw, ground beef met the performance standard; 1.1 and 2% of 19,365 and 17,849 samples, respectively, were positive for *Salmonella*. In 2007, approximately 1,400 USDA-inspected establishments in the US produced raw ground beef that was routinely sampled for *E. coli* O157:H7 as part of the FSIS HACCP verification programs. Progress has been made by US processors in reducing the prevalence of *E. coli* O157:H7 in raw ground beef (Table 26.1).

Over the past few years, FSIS increased its use of the phrase "statistical-based sampling and testing" in their rules, directives, and notices, and communications to the consumers and media. However, FSIS has not fully delineated the statistical limitations of sampling and testing plans, nor linked the limitations to the performance standards, particularly the zero-tolerance performance standards. For example, sampling and testing can be used to screen out some, but not all, lots of raw beef that exceed 1 *E. coli* O157:H7/250 g. Assuming random, homogeneous distribution, and testing 25 g from each of 30 sample units, a negative result provides 95% confidence that the concentration of *E. coli* O157:H7 in the lot is no more than 1 cell per 250 g. This sampling plan ($n = 30$, $c = 0$) has a difficult time detecting positive lots of raw beef where *E. coli* O157:H7 is unlikely to be randomly distributed or homogeneous. Even with the $n = 30$ sampling plan, there is a 74% probability of accepting the lot when the proportion of positive units is 1%, and in general, the prevalence of *E. coli* O157:H7 in raw beef destined for non-intact beef is much lower than 1%. In 2007 FSIS considered a $n = 60$ sampling plan as a best practice for the detection of *E. coli* O157:H7 in raw beef destined for non-intact products and the non-intact products themselves. The plan has significant statistical uncertainty and assumptions and a relatively low statistical probability of detecting low levels of *E. coli* O157:H7 in beef. FSIS should rationalize its zero-tolerance policy based on the heterogeneous and nonrandom nature of *E. coli* O157:H7 contamination and the lack of statistical confidence associated with its regulatory sampling and testing program.

Table 26.1 Prevalence of *E. coli* O157:H7 in US raw ground beef based on FSIS verification testing

Year	Prevalence %	No. of positives/No. of samples
1998	0.17	14/8,067
1999	0.41	32/7,769
2000	0.85	54/6,362
2001	0.84	59/7,004
2002	0.78	55/7,024
2003	0.31	20/6,553
2004	0.18	14/7,994
2005	0.16	18/10,961
2006	0.17	20/11,759
2007	0.24	29/12,230

ICMSF (2002) stated that food safety management systems based on preventing hazards through GHP and HACCP are much more effective in ensuring safe foods than is end-product testing. In fact, these international experts expressed concern over the continued indiscriminate use of microbiological testing of the end product. ICMSF concluded that microbiological testing can be useful in the management of food safety, but tests should be selected and applied with the knowledge of their limitations. Testing, whether by USDA or by industry, has been presented by FSIS as a significant means to prevent microbiological hazards from reaching the consumer. This can be misleading and causes overreaction by regulatory authorities, legislators, and consumer activists when tests for pathogens come back positive, particularly on raw meat products.

Risk-Based Inspection

In 2007 there was a major attempt to develop and apply the concept of RBI by the US Under Secretary for Food Safety. The Under Secretary stated that RBI has the potential to change inspection by FSIS from uniform inspection independent of product risk and establishment risk control measures to RBI based on differences in inherent risk measure and performance of establishments in controlling risks. In theory, this should reward establishments demonstrating a high degree of risk control with less USDA inspection and sampling. In contrast, enhanced inspection activities would occur in those establishments where risk control is lower and for those products where inherent risk is higher. The RBI process requires the analysis of all inspected products for their inherent risk based on such factors as whether there are cooking steps, antimicrobial treatments, or post-lethality exposure to food contact surfaces. The RBI process also means that all establishments must be evaluated for risk control based on factors such as adequately designed HACCP systems, regulatory compliance history, and use of microbiological sampling and testing to verify system performance. Whatever the criteria used to evaluate risk and control for the purposes of RBI, they should be linked by scientific data to their public health consequences. Most of the food safety stakeholders in the US believe in RBI, at least conceptually, and its application to the entire food supply chain, not only for USDA-regulated products.

A major impetus for RBI began in the mid-1980s through the 1990s when studies conducted by NAS and FSIS established the need for fundamental change in the meat and poultry inspection program. Key changes included the need for FSIS to modernize its inspection processes, make better use of its resources, reduce its reliance on organoleptic inspection, and shift to prevention-oriented inspection systems based on qualitative and quantitative risk assessments. These and other scientific studies and reports paved the way for FSIS to publish its landmark rule, the PR/HACCP rule. Another milestone in the evolution of RBI was the publication, on June 6, 2003, of the interim

final rule for control of *L. monocytogenes*in RTE meat and poultry products (FSIS, 2003). The regulation is based on a risk assessment, albeit with many assumptions and uncertainties, and provides establishments with different options to control contamination in order to produce safe, unadulterated product. FSIS bases its verification activities on the interventions that an establishment chooses to adopt and on the potential for *Listeria* growth in products produced at that establishment. Based on the perceived success of the *Listeria* initiative, in February 2006, FSIS announced an 11-point program representing a risk-based strategy for *Salmonella*. The initiative included concentrating resources at establishments with less effective controls and hence higher levels of *Salmonella*.

FSIS continued to refine the RBI concept in 2007 and 2008 using inputs from a series of public meetings, an objective third party, and the US National Advisory Committee on Meat and Poultry Inspection. Unfortunately, the RBI effort related to meat processing was slowed because of legislative constraints, stemming from cautionary and reactionary calls from consumer activists and the inspection union that restricted the implementation of RBI in meat processing establishments. However, to circumvent the restrictions regarding RBI in processing operations, FSIS is continuing to refine its algorithm and strategies for what it calls public health RBI systems for slaughter. If it moves forward, important issues related to the successful execution of RBI include the confidentiality of establishment-specific risk rankings; development of product-inherent risk rankings; measurement of the effectiveness of RBI by FSIS, industry, and other stakeholders; use of volume in predicting risk; use of records such as NRs to affect the RBI score; and management of misunderstandings and disagreements related to RBI to continuously improve the process. To develop and implement a system for risk-based allocation of inspection resources will require cooperation among all stakeholders, transparency with FSIS, and legislative endorsement.

Food Safety Research on Meat Hygiene

Government contributions to the food safety of meat products would be enhanced through a focus on science-based interventions, applied research, and education initiatives and not on punitive measures directed at industry, which will have a negligible impact on maintaining or improving the safety of the food supply or improving public health outcomes. Each fiscal year the US Congress appropriates funds to federal agencies to carry out their functions, including funds that are directed for research programs (Table 26.2). The House and Senate Appropriations Subcommittees on Agriculture have preliminary jurisdiction over the funding that is approved for research activities conducted by the USDA and its departments.

Table 26.2 Total USDA/ARS and USDA/CSREES funding for food safety research

Year	USDA/ARS funding ($)	USDA/CSREES funding ($)
2000	1,000,000	No data
2001	1,750,000	289,000
2002	1,990,000	800,000
2003	2,340,000	900,000
2004	2,340,000	805,000
2005	2,639,000	900,000
2006	2,739,000	1,000,000
2007	2,739,000	1,000,000
2008 – projected	2,873,339	990,000
Cumulative total	20,410,339	6,684,000

In 2005 lobbyists and scientists from AMI were successful in increasing funding for food safety research related to *E. coli* O157:H7,*L. monocytogenes*, and *Salmonella* in meat and poultry products to totals exceeding $2.6 million for USDA's Agricultural Research Service (ARS) and $900,000 for USDA's Cooperative State Research, Education and Extension Service (CSREES). Since 2000, total funding provided has been nearly $20 million for ARS and $7 million for CSREES. What is needed is a cohesive, well-defined research plan to reduce the incidence of foodborne pathogens in meat products. The plan needs measurable milestones, transparent oversight, cooperation, and participation by all stakeholders and both short- and long-term objectives. Based on results to date, US taxpayers may not be getting the optimal returns on their tax dollars allocated for USDA-directed meat hygiene research.

Inspection Resources for Ensuring Meat Hygiene in the US

In the US, food is regulated by at least 12 federal agencies and 35 different statutes. Ensuring the safety of the US food supply is a challenge with over 40,000 food manufacturing and processing establishments, over 110,000 food retail establishments, and over 930,000 restaurants, plus the international businesses exporting to the US. Under the FMIA, PPIA, and the Egg Products Inspection Act (EPIA, 21 USC §§ 1031 *et. seq.*), FSIS issues and implements regulations governing the production of meat, poultry, and certain egg products prepared for distribution in commerce. FDA monitors all other food products according to the FFDCA. The US Government Accounting Office (GAO, 2004) stated that the "federal food safety system is not the product of strategic design." The disproportionate allocations of resources between the two primary agencies, FSIS and FDA, charged with ensuring the safety of the food supply are extreme. FDA has the responsibility for ensuring the safety of about 79% of the foods consumed by US consumers, but does this with only about one-third

(about $450 million) of the total US food safety budget. This is in contrast to FSIS, where about two-thirds (about $900 million) of the total budget is spent to inspect about 21% of the food supply. According to CSPI (2007b), outbreaks from foods regulated by FDA (e.g., seafood, produce, and dairy products) account for 67% of the more than 5,300 outbreaks in their database and resulted in over twice the number of illnesses as that caused by foods regulated by USDA. The disparity also applies to the human resources in that FSIS has about 7,600 inspectors for daily oversight of about 7,021 meat, poultry, and egg product processing and slaughter establishments; in contrast, FDA has only about 1,900 food inspectors for about 210,000 food establishments. CSPI (2007b) pointed out that FDA inspects the food establishments for which it has responsibility just once every 10 years, in contrast to daily inspection at meat and poultry slaughter establishments and most processing establishments.

According to CSPI (2007a), the average US citizen eats about 118 kg (13% of the total intake) of imported foods annually. USDA has an equivalency requirement for exporters to the US, legislated by the US Congress, whereby both the national food safety programs and meat processing establishments must be approved, and essentially 100% of shipments (about 1.8 billion kg of meat and poultry and 2.7 million kg of liquid egg products) are visually checked upon entry into the USA. Because of limited resources and decisions on priorities, FDA inspects less than 1% of the imported food shipments under their jurisdiction.

The GAO (2004) and CSPI (2004, 2007a,b) have called for changes in the manner in which the federal government regulates the food industry, recommending an overhaul of legislation to create a uniform, consistent, and risk-based food safety policy and a consolidation of all food safety agencies to improve the effectiveness and efficiency of the federal food safety system. CSPI recommended that a single, independent food safety agency, administering a unified statute, could better address the problems with food safety inspection and regulation, including gaps in consumer protection, inadequate coordination, conflicting public health standards, regulatory redundancies, and slow approval of new technologies.

Members of the US Congress also have called publicly for a unification of the US food safety system; bills introduced in 2007 included the Safe Food Act, Consumer Food Safety Act, Assured Food Safety Act, Food Import Safety Act, and Food and Product Responsibility Act. Thus far, the translation of legislative grandstanding into meaningful action has not occurred; this likely is beneficial since none of the bills offer a truly thoughtful approach to a unified food safety system, developed transparently using all stakeholders and taking into account the changes that must occur in the FMIA, PPIA, EPIA, FFDCA, and other laws before meaningful progress can be made. For the benefit of the entire US food system, the unified food safety agency would implement RBI and take one approach to the development and implementation of food safety criteria, including performance standards. Many stakeholders would argue that the barriers in the US to such a paradigm shift are too numerous and too great to overcome.

Science-Based vs. Politically-Based Regulations

Although FSIS speaks often of science-based regulatory policies for the meat industry, there are numerous examples where FSIS policies appear to be driven more by politics than science. When US meat hygiene regulations are not science based, it leads to challenges of meeting politically driven expectations in a world where scientific principles play a major role in the presence of hazards and their impact on human health. Sperber (2005) characterized the government approach to science-based HACCP regulations and performance standards as "opaque legislation-based systems," in contrast to "transparent science-based systems." Sperber cited a rule-making process that is not transparent, noting the regulators' ignorance of HACCP principles and implementation, and the inappropriate use of statistics in the name of science as reasons for the opacity.

One of the major challenges facing the USDA in maintaining a science-based approach to meat hygiene policies is that many of the leadership positions in USDA and FSIS are filled as political appointees; thus, those who are in a position to serve the US public also are pressured by political incumbents to address meat hygiene based on political interests. Consumer groups that actively engage the US Congress on food safety issues expect the political appointees to support their position or risk being labeled as "anti-food safety." In a political world of sound bites and rhetoric, it is difficult for any member of the US Congress to be against "improving food safety," even though there are many different means to accomplish improved meat hygiene.

Another complicating factor influencing the implementation of science-based policies is that most of the 7,600 USDA inspectors that observe meat hygiene practices are unionized and, as such, are protected from individual scrutiny and measurement, except presumably by a small circle of federal human resource staff. It is essentially impossible to obtain data on inspector performance, individually or collectively. There is no means to determine how many regulatory citations are issued by the average inspector and individual inspectors, in order to better understand which inspectors are at the extremes of such a histogram. There is no means to measure inspector competency and no means to assess the consistency of inspection across the US meat industry. The unionized employees appear to be politically connected and protected.

There are examples in 2007 where FSIS regulatory activity appears to be influenced greatly by social and political pressures even though FSIS purports that it is an agency driven and guided by science. The two issues discussed briefly herein to exemplify political influence over science-based policies are the use of trace amounts of carbon monoxide (CO) in modified atmosphere packaging (MAP) systems and the use of irradiation for meat products.

The FDA review process for ingredients and additives that are generally recognized as safe (GRAS) provides a means for evaluating the safety of ingredients and additives used in food production and processing. Likewise, the USDA process for label review of USDA-regulated items provides a process

to ensure consumers of the suitability of an ingredient or additive for use in USDA-inspected meat products. MAP systems, like those that use trace amounts of CO, provide many consumer and marketing benefits and have been used safely in food and meat packaging for decades, e.g., for pre-mixed salads, potato chips, nuts, bakery products, coffee, pasta, shredded cheese, and pre-cut vegetables.

In February 2002, FDA provided a favorable GRAS response letter to a petitioner and maker of a meat packaging system that used trace amounts of CO in a low-oxygen MAP to stabilize color and preserve the product. Shortly afterward, USDA evaluated the system for suitability and provided a means for companies to use the packaging system as proposed. Two subsequent petitions dealing with low-oxygen MAP with CO were favorably reviewed by FDA and USDA with the same result.

As more meat firms adopted this packaging technology, less were using its precursor that relied on high-oxygen MAP environment and the incorporation of a rosemary extract as an antioxidant and color stabilizer. Consequently, one of the firms that produces rosemary extract decided to challenge the FDA and USDA conclusions on the low-oxygen systems and embarked on a media and lobbying campaign to discredit the low-oxygen system. Specifically, in November 2006 a company that manufactures rosemary extract filed a petition with FDA challenging the GRAS status of all of the previously determined packaging systems that use trace amounts of CO and requested that FDA, and hence USDA, terminate the use of CO in fresh meat packaging systems.

Some allegations about this MAP system falsely claimed that it masked spoilage. In a June 2004 letter, FSIS provided the opinion that the use of approved MAP systems with trace amounts of CO for use with case-ready fresh cuts of meat and ground meat did not mislead consumers into believing that they are purchasing a product that is fresher or of greater value that it actually is or increase the potential for masking spoilage.

During consideration of the National Food Uniform Labeling Act of 2006, an amendment was sought by the Congressional representative from the home state of the company manufacturing the rosemary extract to limit the industry's ability to use MAP that uses trace amounts of CO. Despite the defeat of the amendment, legislative efforts continued to keep the issue alive in an attempt to prohibit the use of CO in MAP systems. This exemplifies how a politically based agenda can affect regulation in the meat industry despite the absence of a scientific rationale.

A second example of recent meat hygiene regulatory activity that is not linked to science is the use of irradiation to improve the safety of meat products. FDA, the primary regulatory agency overseeing the approval of irradiation in the US, approved irradiation for use in fruits, vegetables, spices, grains, raw poultry, raw pork, and raw beef. Although RTE products may also benefit from irradiation processing, FDA has not yet promulgated rules to permit the use of irradiation for RTE meat and poultry products.

In December 1997, FDA issued a final rule authorizing irradiation of meat to control foodborne pathogens and extend product shelf life. In August 1998, FDA amended the December 1997 rule to allow more flexible labeling of irradiated products. In March 1999, FSIS published a proposed rule and in December 1999 a final rule implementing the FDA regulations and permitting irradiation of raw meat. The final rule required use of the radura symbol, and irradiated products were required to bear a label declaration identifying the products as irradiated. The FDA and FSIS final rules, however, do not permit irradiation of meat and poultry containing ingredients other than dry spices (e.g., marinated pork chops, wieners, RTE products, or bacon), thus diminishing the opportunities to improve food safety.

In August 1999 a coalition of food industry trade associations, health organizations, and academic and consumer groups filed a petition asking FDA to extend the use of food irradiation to RTE meat and poultry products, and fruit and vegetable products. The petition documented the safety and wholesomeness of irradiated RTE meats, poultry, fruits, and vegetables and assessed the impact on relevant essential nutrients in those foods, concluding that the nutrient reduction would be negligible. The petition also requested expedited review by FDA. FDA accepted the petition in October 1999 and determined that there were additional data needs on certain non-meat RTE products. To date, a timeline for completion of this review has not been provided.

In October 2002, AMI sent a letter to the Secretary requesting that the USDA Agricultural Marketing Service (AMS) establish a pilot program for purchasing irradiated ground beef in the National School Lunch Program (NSLP) commodity beef-purchasing program managed by AMS, thereby reducing the likelihood of exposure of children to bacterial pathogens associated with raw ground beef. In May 2003, USDA announced purchasing specifications for irradiated ground beef through the NSLP for the 2004 school year. Furthermore, the 2002 Congressional Farm Bill directed USDA not to prohibit the use of approved food safety technologies in the NSLP. However, in November 2003, the consumer activist group, Public Citizen, filed a petition with FDA to ban irradiation of beef products. The petition cited a finding in an unpublished study conducted by Public Citizen that irradiated beef products purchased in the marketplace contained 2-alkylcyclobutanones, which have been reportedly linked to cancer cell promotion in lab animals.

In 2003, AMIF, in conjunction with the US National Cattlemen's Beef Board, agreed to fund an exploratory research project to determine the viability of using very low dose irradiation applied to the beef carcass surface to destroy any potentially pathogenic bacteria. FSIS provided guidance on the type of information that they would need to address this application of irradiation. FSIS also gave the preliminary regulatory and legal opinions indicating that this application would be treated as a processing aid; thus labeling of the subsequent products would not be required or appropriate. The AMIF–Cattlemen's project regarding pathogen reduction and organoleptic properties of surface-irradiated product demonstrated clearly that the treatment was effective at reducing *E. coli*

O157:H7 on surrogate carcass surface pieces and created no measurable impact on organoleptic properties, shelf life, or on the nutritional profile. The project results were submitted to the FSIS New Technology Office for review. Unfortunately, while agreeing that the data had satisfactorily addressed all issues raised in the original FSIS review, the New Technology Office determined that the minimal surface treatment would require labeling of the secondary products produced from the carcass. Subsequent to this decision, FSIS policy staff rescinded the original approval and requested that a petition be submitted to FSIS; such a petition was under review at FSIS for over one year with no apparent action. For an organization that purports itself as a public health agency, FSIS has moved very slowly to promote publicly the irradiation intervention and to provide opportunities for industry to take advantage of irradiation at the carcass level and for RTE meat products.

Conclusions

During the past two decades, FSIS has tried unsuccessfully to transition from an inspection agency to a public health regulatory agency. This is not their charge as defined by the FMIA; however, FSIS prefers to speak in terms of protecting public health rather than educating, inspecting, and improving the meat sector. After all, it is difficult for the US Congress, the White House, consumers, and any stakeholder to be against *improving public health*.

Consumers expect that RTE foods are safe to eat; and consumer education on safe methods for handling and preparing raw foods cannot be overemphasized if the safety of these foods is to be assured. Although it is not always a popular issue to raise, regulators and industry must face the business realities that there will be a point at which further reductions in risks associated with specific foods may have additional costs that are unreasonable, despite what some stakeholders proclaim. Regulatory authorities can afford to be conservative as they do not bear the direct costs of tightened performance standards nor are they required by law to demonstrate that the performance standards are technically achievable and effective in significantly improving public health.

FSIS should increase its cooperative role with industry in achieving the goals of reducing the prevalence of pathogens in meat and in reducing the public health risks from meat food products. FSIS should credit industry with some of the success in reducing the prevalence of potential pathogens. If one were to only read the FSIS reports, one would conclude that it is only because of the regulatory enforcement that the prevalence of pathogens such as *Salmonella* is decreasing. For example, after stating that the rate of *Salmonella* in raw meat and poultry dropped by 66% over the past six years and by 16% compared to 2002, FSIS claimed that the "declining figures demonstrate that strong, science-based enforcement of food safety rules is driving down the rate of *Salmonella*" (*Salmonella* Incidences, 2003). One must question the accuracy of the phrase

"science-based enforcement"; such positioning reflects an apparent need by FSIS to reflect an enforcement attitude against an uncooperative industry. FSIS and its many stakeholders would be better served if FSIS communications reinforced a successful working partnership with all stakeholders to optimize pathogen reduction and improvement in public health.

The federal agencies regulating the food supply can take advantage of enormous financial resources provided by US citizens to redefine a more transparent and cooperative approach with all stakeholders to establish and achieve mutual food safety goals and objectives. The federal government can acknowledge the limitations of the current multi-agency food safety organization and recommend changes, consolidation, and cooperation that will deploy the financial and human resources against the prioritized risks facing the food industry and US consumers, without regard to preconceived boundaries. The regulatory agencies governing the food industry need leadership from legislative and administrative offices to make the paradigm shifts.

Industry must continue its effort to continuously improve their operations to further reduce risks associated with food production. It must use its collective scientific expertise, and that developed through cooperative relationships with academia and government researchers, to seek new solutions to food safety risks. Industry must recognize that governmental regulatory agencies will need constant attention to affect their rule-making processes and outcomes. Industry must operate in an environment of legislative and regulatory HACCP, and their associated performance standards, until the agencies tackle the challenging issues described herein. The direction for improving the use of performance standards will be focused when the foodborne disease surveillance system defines clearer links between specific food products and foodborne illnesses. Statistically significant prevalence and epidemiological data will help facilitate the prioritization of food safety risks, for meat and non-meat food products, across the entire food supply chain from production to consumption. Establishing measurement systems that allow government and industry to determine progress against food safety goals, without a pre-designed negative consequence for industry working toward continuous improvement, will lower food safety risks.

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