ADVANCES IN DAIRY MICROBIAL PRODUCTS

Edited by
Joginder Singh
Ashish Vyas

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Advances in Dairy Microbial Products

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Chapter 1

Global scenario of fermented dairy products: current advancements and future challenges

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1.1 Introduction

Fermented dairy products are popular because of the high nutritional value and health promoting properties including alleviation of numerous health conditions. This valuable health promoting attribute is imparted by peptides and exopolysaccharides (EPSs), which have bioactive properties such as hypocholesterolemic, antimicrobial, antioxidative, and immunomodulatory activities. The strains which produce such peptides and EPSs are being studied for incorporation into fermented dairy products. There is also a need to relate industrially important characteristics of the lactic acid bacteria (LAB) with their genomic characteristics in order to exploit their metabolic potential to the optimum level. The LAB strains which are commonly used in fermented dairy products have attributes which can be exploited with the application of metabolic engineering and genome scale metabolic models (Stefanovic et al., 2017). The advances in genomic studies of LAB include improvement in flavor, texture and prevention of phage attacks. Microencapsulation technologies are being suggested as the future of probiotics, to ensure safe passage through the gastrointestinal tract (GIT) of viable probiotic bacteria. The trend of cultured bio-yogurts or cultured probiotic milk and probiotic beverages is the potential solution for lactose intolerant people. The future of fermented dairy products also includes advancements in packaging such as the use of sensors; nanotechnology to device smart packaging technologies to maintain viability of the probiotic strains present in the fermented dairy product.

1.2 Bioactive peptides in fermented milk products

Fermentation of milk by starter and nonstarter microorganisms release inactive peptides from the parent milk proteins and transform them into physiologically and functionally active forms (Park & Nam, 2015). Bioactive peptides (BAPs) released in fermented dairy products have been known to exhibit specific bioactivities which alleviate numerous health conditions. Various fermented milks and cheese varieties from all over the globe are products with naturally derived BAPs. Commercial fermented dairy products are now supplemented with BAPs derived from milk proteins. The trend of including multifunctional BAPs in fermented dairy products will pick up in the future; milk proteins are considered the best source of BAPs, and starter cultures can transform the milk proteins into BAPs (Tagliazucchi et al., 2019). Most of the bioactive peptide formation in dairy fermentation is obtained from LABs belonging to the genus *Lactobacillus*. Lactobacilli are fastidious Gram positive bacilli with auxotrophy for several amino acids. In order to compensate for their amino acid requirements, these bacteria hydrolyze proteins in their surrounding environment to release peptides and amino acids (Stefanovic et al., 2017). The lactobacilli have a proteolytic system with an enzyme complex called cell envelope proteinases (CEPs) to hydrolyze milk proteins. The peptides released contribute to the organoleptic properties of the fermented milk product and also exhibit various remarkable biological functions (Griffiths & Tellez, 2013).

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BAPs are protein fragments or peptides, size ranging from two to twenty amino acid residues. They are obtained from dietary proteins upon hydrolysis by digestive enzymes in the GIT, microbial metabolism of the proteins during food fermentations, or proteolytic digestion by microbial or plant derived enzymes (Venegas-Ortega et al., 2019). These peptides, depending upon the amino acid sequence, are known to have a positive impact on human health. They are reported to exhibit antimicrobial, antioxidative, opioid, antidiabetic, satiating, antithrombotic, antihypertensive, immunomodulatory, mineral-binding, angiotensin converting enzyme (ACE) inhibiting, and anticariogenic activities (Nongonierma & FitzGerald, 2015).

The desirable attributes of milk derived biopeptides are stability at acidic and alkaline pH and resistance to digestive enzymes. The first BAPs reported were the caseinophosphopeptides (CPPs), claiming to enhance bone calcification in infants. Some examples of BAPs are ACE (angiotensin I-converting enzyme)—inhibitory peptides; the lactotripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), identified in milk fermented with *Lactobacillus helveticus*; CPPs and whey derived peptide. IPP and VPP are reported to be the most potent antihypertensive biopeptides (Rutella et al., 2016). CPPs potentially enhance mucosal immunity and prevent osteoporosis, dental caries, hypertension and anemia. Milk derived opioid BAPs are β-casomorphins, exorphins, α-lactorphin and β-lactorphin (Nongonierma & FitzGerald, 2016). BAPs derived from milk proteins hold promise for development of health-promoting nutraceutical and functional fermented dairy products in the future. An example of such a product is "Calpis," a fermented milk containing the tripeptides VVP and IPP developed using *L. helveticus* CP790 and *Saccharomyces cerevisiae*. Some BAPs relieve oxidative stress due to their antioxidant property. Studies have revealed that obligately homofermentative lactobacilli and heterofermentative *Lactobacillus* strains exhibit antioxidative activity. They exhibit metal ion chelating capacity, reactive oxygen species (ROS) scavenging activity, and oxidative enzyme reducing activity. Examples of LAB with said property are *Lactobacillus acidophilus, Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium longum* (Pihlanto & Korhonen, 2015).

The antimicrobial BAPs (AMPs) are considered as promising constituents of health-enhancing nutraceuticals. AMPs are expressed constitutively upon infectious stimuli from bacteria or bacterial components. As these AMPs are small sized, they are rapidly secreted outside the cells and thereby provide quick response to pathogenic bacteria, fungi or parasites.

Some examples of LAB with proteolytic system for release of AMPs are *Lactococcus lactis, L. helveticus* and *Lactobacillus delbrueckii* var. *bulgaricus* (Mohanty et al., 2016). The AMPs are effective against bacteria (*Escherichia, Helicobacter, Listeria, Salmonella*, and *Staphylococcus*), yeast, and filamentous fungi. Casein protein derived AMPs are kappacin, casecidin and caseicin. Kappacin forms pores in target cell membranes to kills pathogens and casecidin is effective against a broad spectrum of pathogens. The AMP, Lactoferricin is derived from whey protein lactoferrin which is effective against several gram-positive and gram-negative pathogens (*E. coli, Listeria monocytogenes*), viruses and fungi (Park & Nam, 2015).

Several strategies are employed to enhance BAP production from milk proteins to develop multifunctional fermented dairy products. These include exploiting proteolytic enzyme system of LAB, use of food grade proteolytic enzymes or supplementing fermented milk products with synthesized BAPs (Hafeez et al., 2014).

Raveschot et al. (2018) described selection of BAP producing strains of Lactobacilli for development of a multifunctional fermented dairy product. The strain selection criteria are based on the potential of bioactivities of the peptides it produces. A combination of conventional methods and in-silico methods are used for the purpose (Punia et al., 2020). The proposed method begins with genotyping of CEPs. Each species exhibits different proteinase content and the PCR method is used for genotyping and sequencing of CEPs. Strains can be grouped depending upon the CEP genotypes present in them. Substrate selection is done from peptide databases available (Nielsen et al., 2017). The resulting peptides are checked for heterogeneity using mass spectrophotometry (Ms), statistical and bioinformatics tools (Tu et al., 2018). The most promising hydrolysate will be selected based on its in-vitro bioactivities. Peptide identification and its activity allocation is possible from BAP databases and quantitative structure activity relationship (QSAR) modeling (Nielsen et al., 2017). QSAR models enable the establishment of correlations between structural characteristics of peptide molecule and its bioactivity.

Recent advances in the search for BAP-producing strains include development of computational tools and software like, PeptideCutter software which help to mimic peptide formation in proteolysis and calculate amino acid sequence in predicted peptides. Such type of predictions can help in generation of BAPs from a precursor with suitable enzymes. Software PeptideLocator is used for prediction of BAPs in protein sequences (Mooney et al., 2013). ToxinPred software is used for prediction of undesirable toxic peptide sequences if any (Gupta et al., 2013).

Bulk production of BAPs can also be done for use in development of functional foods. Even though the BAPs in dairy products are not as potent as drugs for ailments like hypertension, they add extra-nutritional attributes desirable in

a diet. The parameters considered for production are the choice of starting material, enzymes for hydrolysis, and the ease of purification process (usually membrane separation). Enzymes used for generation of BAPs are combinations of endoproteinases, including chymotrypsin, pepsin, thermolysin, pancreatin, elastase, carboxypeptidase, and proline-specific endopeptidase. An example is the use of immobilized trypsin in a fluidized bed bioreactor to produce CPP α_s-CN and β-CN (Choi et al., 2012). The methods, which can be adopted for production of BAPs, are exploiting the proteolytic system of LAB or food grade enzymes or combination of both to release the BAPs from milk proteins directly into the fermented milk products, thereby supplementing the fermented milk products with the BAPs or a production of the BAPs by microorganisms using recombinant DNA technology (Hafeez et al., 2014).

Thus, design and development of multifunctional fermented dairy products with biological activities at a commercial scale will be possible with these advances in technology and information. Indigenous fermented dairy products from different parts of the world may prove to be potential sources of novel BAPs. Exploring these spontaneous milk fermentations with indigenous strains may yield several more industrially important strains and BAPs.

1.3 Advances in the genomics and metabolomics of dairy lactobacilli

The role of LAB in fermented dairy products is well documented. The advances in the application of LAB involve the study of genetic characteristics which influence the development of unique flavor (Van Kranenburg et al., 2002), confer phage infection resistance (Mahony et al., 2016), or release of BAPs (Park & Nam, 2015; Raveschot et al., 2018) and EPSs (Wu et al., 2014). Based on the genomic, proteomic, and metabolomics data, mathematical or computational models can be developed to enable the prediction of growth and robustness in performance of LAB under specific conditions (Stefanovic et al., 2017). This concept is called metabolic engineering and it can be used to obtain fermented dairy products with desirable organoleptic, nutraceutical, functional and technological properties.

One of the major problems associated with dairy industries causing failure of fermentation and economic loss is phage infection of domesticated cultures of LAB (Fernández et al., 2017; Mahony et al., 2012). LAB−infecting phages belong to the *Siphoviridae*, *Myoviridae*, or *Podoviridae* family. Phage infection of starter cultures in fermentation leads to low acid production, inconsistent organoleptic properties, and often fermentation failure (Mahony et al., 2016). Conventionally used strategies for phage control in dairy fermentations included maintenance of sanitation, raw material treatment, rotation of starter cultures, strain selection, use of chemical control agents like sodium hypochlorite, peracetic acid (Fernández et al., 2017), bacteriophage-insensitive mutants (BIMs), and design of plasmids with phage-resistance. However, the efficiency of some of these measures depend on phage susceptibility, initial load of phage population and medium in which phage is present. Some of the LAB bacteriophages are also known to adapt to high temperature and disinfectant treatments (Giraffa et al., 2017).

The discovery of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) based technologies has revolutionized the study of phage infection and prevention in LAB (Barrangou, 2014; Stout et al., 2017). It was originally discovered in the intergenic region adjacent to the alkaline phosphatase (iap) gene of *E. coli* in 1987. CRISPR-Cas systems consists of a CRISPR array and *cas* genes which function as adaptive immune systems in prokaryotes and archaea that provide protection against infection by viruses and other mobile genetic elements. (Sanozky-Dawes et al., 2015). These arrays are the basis of genetic memory of the bacterial adaptive immunity. These diverse systems are classified into two classes, six types, and 19 subtypes based on the *cas* genes and mode of action (Barrangou & Horvath, 2017). The common features in all classes include a CRISPR locus, variable spacers, and a set of associated *cas* genes with the ability to execute specific DNA cleavage (Westra et al., 2016).

A typical CRISPR locus contains a series of DNA repeats and repeat spacer arrays (Stefanovic et al., 2017). Spacers are short DNA sequences of foreign DNA captured from invasive viral DNA during exposure. Spacers thus maintain a record which can be considered as a genetic barcode for each type of viral exposure for the bacterial strain (Barrangou & Horvath, 2017). The acquisition of invasive DNA in the CRISPR loci is attributed to the efficient defense mechanism towards the foreign viral DNA. When the virus attacks the LAB, the invasive DNA from CRISPR loci is transcribed into CRISPR RNA which eventually cleave the incoming matching foreign DNA. Thus, CRISPR maintains a record of previous phage infections and carves a genetic memory against future infections, thereby, providing adaptive immunity against phages (Barrangou, 2014).

CRISPR-Cas-(CRISPR-associated protein) based technologies has brought a paramount change in genome editing of LAB in the quest for robust industrial strains (Zhou et al., 2019). CRISPR-Cas systems are used to study the interactions between LAB and their viruses. The first successful industrial application of CRISPR-Cas systems was carried out on *S. thermophilus* wherein phage DNA was incorporated in its CRISPR array to achieve phage resistance (Levin et al.,

2013). This is done by challenging the bacterial strain with the phage attack and making them develop immunity. Such technology is highly valuable for development of phage resistant commercial starter cultures.

Millen et al. (2012) described a novel Type III, self-transmissible, plasmid-encoded, phage-interfering CRISPR/Cas system for the first time in *Lactococcus lactis*. A CRISPR-Cas type II-A system in *Lactobacillus gasseri* was characterized for genome editing and the study of phage infection (Sanozky-Dawes et al., 2015). There are now 1,262 publically available *lactobacilli* genomes containing CRISPR-Cas adaptive immunity. Common probiotic species with CRISPR-Cas systems present in them include *L. acidophilus, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus fermentum, L. gasseri, Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus mucosae, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus*, and *Lactobacillus salivarius. These* CRISPR-Cas systems *enable precision genome editing for use of probiotic bacteria as live therapeutics*. Besides conferring phage resistance, CRISPR-Cas systems can also be utilized for genome enhancement to impart desirable probiotic characteristics like survival in GIT, temperature tolerance, oxidative stress tolerance, osmotic stress tolerance, colonization of host, resistance to acid and bile and uptake, catabolism of prebiotics, and enhanced functional properties (Stout et al., 2017).

Flavor is one of the most significant characteristics of any fermented dairy product. Flavor of a fermented dairy product is influenced by the natural population of complex fermenting microbiota and their metabolic end products released during natural or spontaneous fermentation. On the other hand, defined starter cultures, especially members of LAB, adjunct cultures, and their metabolic end products are responsible for flavor in industrial fermentations (Mills et al., 2010). Besides the LAB, substantial contribution to the flavor comes from other non-LAB species, certain yeasts, and some molds. The generation of flavor compounds arise from metabolism of sugar (glycolysis), protein (proteolysis) and lipid (lipolysis) by the microbiota involved in fermentation. For thousands of years, flavor development was solely contributed by the metabolism of the microbes involved in fermentation of the milk substrate and it would suffer variation according to population dynamics. With the advances in information available on flavor contributing compounds and their biochemical pathways, it is now a possibility to improve and maintain the consistency in flavor of a product (Wu et al., 2017). Advances in genomic and metabolomic studies have made it now possible to exploit specific bacterial strains and design them to obtain desirable flavors. Amino acids are the precursors of several flavor compounds in fermented milk products. As the LAB are obligate fermentative organisms, they do not possess the complete tricarboxylic acid cycle (TCA), amino acid metabolism pathways are incomplete and also vary among species. Recent sequencing of complete genomes has provided much needed information on the enzymes involved in flavor forming metabolic pathways (Gómez de Cadiñanos et al., 2013).

Liu et al. (2014) described the reverse pathway engineering (RPE) approach which uses chemo-informatics and bioinformatics to study enzymes, a flavor forming biochemical pathway between precursor amino acid leucine and formation of 3-methylbutanoic acid from alpha-hydroxy-isocaproate along with synthesis of dimethyl sulfide in LAB. In this method, a desirable flavor compound is selected and its structure is obtained from a database such as NCBI PubChem database (http://pubchem.ncbi.nlm.nih.gov/). Then, a reaction database, BioPath.Database (Molecular Networks GmbH) (http://www.molecular-networks.com/databases/biopath.html), is used to predict flavor forming pathways based on structure submitted. The result from the reaction pool will be several thousands of transformation steps. Next, software called THERESA (THE REtroSynthetic Analyser) helps to select the unknown pathway involved, wherein biotransformation rules for the predicted reactions is used in reverse direction to lead to the metabolic precursor. Bioinformatic analyses will then predict the enzymes that catalyze these reactions. Thus strains with desirable flavor forming pathways will enhance dairy fermentations.

Cheesomics is the use of metatranscriptomics, metaproteomics and metabolomics to study cheese ripening, quality, texture, and flavor of cheese (Afshari et al., 2018). The flavor of cheese depends on the complex dynamics of microbiota involved in processing, ripening, and variations results in the final product because these are the least controllable steps in cheese production (De Filippis et al., 2016). The integrated multiomics approach has helped to overcome hurdles associated with earlier culture-dependent study of microbial interactions and made possible the functional and taxonomic profiling of cheese microbiota. The holistic approach of systems biology involves inclusive analysis of total gene products, *viz*., mRNA transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics) to gain information on the complex microbiota involved in cheese-making (Luca et al., 2018). It has, today, enabled determination for diversity of the cheese microbial community, understand the impact of ripening on flavor, aroma, quality, shelf life, and safety of cheese.

Genome analysis along with physiological characterization of LAB such as *S. thermophilus* is used to investigate the diversity of strains and to provide a reference for selection of novel outstanding strains for use in milk fermentation. Some of the desirable attributes targeted are milk acidification ability, antibiotic resistance, EPS production, γ-aminobutyric acid (GABA) production ability, and survival capacity in the GIT. Some examples of comparative

phylogenetic analysis done among the LAB are *Lactobacillus acidipiscis* found in cheese (Maria et al., 2018), *L. casei*, and *L. helveticus* (Fontana et al., 2018, 2019).

Earlier, the conventional approaches of strain improvement like random mutagenesis, dominant selection, directed evolution, or adaptive evolution and screening were used for selection of improved strains of LAB. This has now been updated by whole-genome sequencing for comparison of LAB strains where new insights are revealed on genetic variations that affect the desirable phenotypes. The targeted phenotypical characteristics are acidification rate, robustness to environmental stresses and phage resistance, contribution to flavor and texture formation, bio-protection activity, and probiotic function (Hill et al., 2017). Metabolic engineering has made it now possible for addition of improved phenotypic characteristics to LAB strains. Some of the methods used for improving LAB strains are Adaptive Laboratory evolution in combination with metaomics analysis for characterization of mutants (Luca et al., 2018); Systems biology tools for elucidating microbial interactions and metabolic capacities; detailed analysis on metabolic flux regulation for LAB model strain—*L. lactis* and metabolic engineering of *L. lactis* as a novel microbial cell factory (Liu et al., 2019).

Adaptive laboratory evolution is used for the study of the physiology of LAB under heat, salt and acid stress, and nutrient starvation conditions in diverse manufacturing conditions like Gouda and cheddar cheese production or ripening. Adaptive laboratory evolution is based on the genomic adaptation to specific environmental stresses or metabolic stress and its effect on phenotypical characteristics. It involves application of transcriptomics, proteomics, and metabolomics followed by physiological characterizations of the strains (Bachmann et al., 2017).

Systems biology tools like Genome-Scale Metabolic models (GEMs) with COnstraint-Based Reconstruction and Analysis (COBRA) are used to predict the nutrient requirements for growth and metabolic patterns for LAB under different conditions. This helps to depict the metabolic end products and their influence on the final product for which the LAB are used (Wu et al., 2017). Thus, the genetic data gives way for the biochemical and physiological information of the LAB strains, and predictions on increased biomass or shifts between homolactic or mixed-acid acid fermentations is made possible with the help of these biology tools.

The production of cheese and yogurt is generally carried out with the help of mixed-starter cultures of LAB. This practice is advantageous as the problem of fermentation failure due to phage infection is prevented. As there is a mixed population of starter cultures, the strain specific phage does not infect all the different types of strains responsible for the fermentation (Smid et al., 2014). The broad range of metabolic activities and the end products of these mixed starter cultures confer the unique flavors and distinct organoleptic properties to the fermented milk products. An insight to the community-level genomics and community dynamics of the mixed consortia is necessary to understand how these unique flavors and characteristics develop. The systems biology approach using high-resolution surveillance of the microbial community dynamics or construction of community-based GEMs will help to study such mixed strains and their role in milk fermentations. It will provide information on growth, nutrient requirements, metabolism, physiology during cheese manufacturing, and ripening (Ercolini, 2017). An example is accelerating cheese ripening by increasing the temperature which in turn increases the biochemical transformations in ripening. This is applied from the metatranscriptomics (RNA-seq) information that expression of genes involved in proteolysis, lipolysis, and amino acids/fatty acids catabolism are promoted in nonstarter LAB at higher ripening temperatures (De Filippis et al., 2016).

Advances in genomics of LAB also include the safety assessment of LAB used in food fermentations. The undesirable qualities of LAB are antibiotic resistance, virulence factors, and production of biogenic amines. With comparative analysis of whole genome sequences, it is possible to detect genes which are related to aforementioned traits. Zhang et al. (2012) performed a comprehensive safety assessment of *L. plantarum* JDM using *L. plantarum* WCFS1 as reference genome. It was revealed that the JDM strain possessed 51 antibiotic resistance-associated genes, 126 virulence-associated genes, and 23 adverse metabolism- associated genes. Another challenge is the formation of toxic biogenic amines (BAs) during LAB fermentation. BAs are low molecular weight organic bases produced through decarboxylation of amino acids. BAs are hazardous to consumers and they have been shown to exhibit synergistic toxicity when more than one type are produced. Formation of BAs can be predicted with comparative genomic analyses and these strains can be removed from the consortia of fermenting LAB (Wu et al., 2017).

1.4 Microencapsulation of a probiotic and prebiotic

Coating the beneficial components and entrapping it into polymeric substances is termed as encapsulation. Food grade materials are used as carriers to encapsulate these materials and have found numerous applications in the food industry. Some of them include controlling certain reactions like oxidation, fermentation and color, and odor modulation, increasing shelf life of the food and help sustain or extend release of some beneficial components. Such applications have also been used widely for encapsulation of live cells such as probiotic cell cultures. This is most often done to shield these

cells from unfavorable environments that they might encounter in food or during storage period. Moreover, prebiotic materials like growth promoting factors like antioxidants and growth essential components like fibers are also subjected to encapsulation by many researchers (Zuidam & Shimoni, 2010). These encapsulates also allow the modulation of various functions and develop innovative approaches to functional foods using this technology. Although encapsulation of prebiotic components is pretty conventional, encapsulation of probiotic cells possess various challenges. Primarily owing to their size that ranges in micrometer range and furthers the challenge to keep them alive during the production of encapsulates.

Therefore, special provisions are made to encapsulate these probiotics, most often LAB using microencapsulation. These microencapsulates have a size range of about 0.2−5000 mm and may employ emulsification as an operating technology. Extrusion could be employed to make even smaller encapsulates to as small as 300 mm. This in turn is a plausible solution to the challenge that enables them to keep these cells alive and to claim potential health benefits in dairy products, owing to the changing dynamics of fermented dairy products, such as lactic acid concentration, pH, and the presence or absence of constituents like peroxides and oxygen. Apart from protecting the cells from these components, encapsulation also plays a critical role in making them available in a metabolically active and viable form.

1.4.1 Encapsulation of live cells

The general process of encapsulation consists of three stages. The first stage is where the component to be encapsulated is incorporated in the matrix. If solid, it will have to be agglomerated and, in the case of liquid, either dispersed or dissolved into the matrix material. In the second step, the liquid is dispersed and pulverized onto the matrix. The final step is achieved with the stabilization of the process though physico-chemical processes. Microencapsulation of probiotic bacteria has been studied using this process. Ding and Shah (2007) studied a few strains of LAB and some bifidobacteria species for their bile tolerance and heat survival when microencapsulated. They found that when free probiotic cultures of these organisms were exposed to these conditions they showed lesser viability as compared with that of microencapsulated cells. This proved that microencapsulation is a suitable technology to preserve the viability of the cells in harsh or unfavorable conditions. Similar studies were carried out by several researchers on different species, reporting similar results.

1.4.1.1 Matrices used for encapsulating microorganisms

Various types of materials viz. alginates, gums, carrageenan, chitosan, starch, cellulosic material, gelatin, and some protein complexes, are used to encapsulate live microbial cells. These matrices are chosen based on various properties that they possess that would be essential for encapsulating live cultures and benefit their survival. Following is the brief account of these matrices.

Alginates

These are polysaccharides that are obtained from natural algal sources. It is made up of α-1-guluronic acids and β-D-mannuronic acids, but may vary in composition according to the source. Rowley et al. (1999) reported suitable use of algal hydrogels as encapsulating matrix for live probiotic cells. It is considered as nontoxic, comparatively simpler, cost effective, and biodegradable source. Also, a mixture of alginates with other polymers has been studied and better results were obtained with combining two or more polymers with alginates (Krasaekoopt et al., 2003).

Carrageenan

Carrageenan is a naturally obtained polymer used for various food applications. As carrageenan works in the range of 40°C−50°C temperature range, it could be easily used to entrap live cells that would be able to operate at room temperatures. Moreover, the carrageenan stabilizes with potassium ions to form microparticles. Also, carrageenan helps sustain the viability of live bacterial cells (Dinakar & Mistry, 1994).

Gums

Certain microbial gums like xanthan and gellan gums are polysaccharides used for encapsulating the live cell cultures effectively. Moreover, their mixtures are more effective in encapsulation of probiotic live cells. These gum have shown better resistance to acids as compared to alginate.

Chitosan

Chitosan is a polysaccharide composed of glucosamine subunits. They polymerize in the presence of polyanions and anions forming cross-linked polymers. Chitosans are used with alginates to encapsulate the probiotic cells that simulate the gastrointestinal conditions for the live cell culture. However, some reports suggest negative influence on the LAB (Groboillot et al., 1993).

Starch

Starch is perhaps the most common of all the polysaccharides that is composed of glucose subunit, bonded with glucosidic bond. Certain categories of starch are nondigestible and known as resistant starches; thus, they are useful as a prebiotic component and also simultaneously as an encapsulating agent. It has good enteric characteristics as a delivery polymer, and it serves as an ideal compound to adhere to the surface of the gut. Starches could also be modified to have different metabolic functions (Crittenden et al., 2001).

Cellulose

As it is a nondigestible polysaccharide it provides good protection from the gastric juices to the live cells. Cellulosic compounds such as cellulose acetate phthalate are used effectively to deliver drug compounds inside the intestine (Mortazavian et al., 2008).

Gelatin

Gelatin is yet another gum that develops thermo-reversible gels. Although capable to be used alone, it can also been used with other above mentioned compounds like gellan gum, to form encapsulating agent for probiotic cell culture. These compounds interact with one another based on their isoelectric point to obtain a net positive charge making the interaction stronger (Anal & Singh, 2007).

Protein-based encapsulating agents

Milk proteins are effective delivery vehicles for probiotic cell cultures. Milk protein has good gelation properties and is biocompatible (Livney, 2010).

1.4.2 Probiotic encapsulation in methods

Microencapsulated probiotic cells have shown better resistance to digestive juices and thus have a better chance of survival in the digestive tract. It is anticipated that the organoleptic properties of the encapsulated probiotic-based products does not change and are at par with that of the original products. It is suggested to obtain an encapsulate in a specific size range below 100 mm in order to preserve the sensory properties of such products (Truelstrup-Hansen et al., 2002).

Various methods for microencapsulation of probiotic cell culture are practiced. The methods most popularly used include spray drying, spray freeze drying, and extrusion. In the spray drying method, polymeric matrices such as starches and gum Arabic are used prominently. It is the most rapid and cost effective technology and is thus, adapted for industrial purposes. However, as high temperatures used in spray drying is detrimental to living cells. Certain additives such as trehalose and other thermoprotective compounds could be added to protect the probiotic cells (Sarao & Arora, 2017). In spray freeze drying, benefits of both spray and freeze drying are combined. Droplets consisting of probiotic cells are atomized into liquid nitrogen and dried. This method provides better control over the size of the capsule; however, it is an energy intensive process and costly compared to spray drying (Zuidam & Shimoni, 2010). Extrusion is a cheaper method used to encapsulate probiotic cells. Carrageenan and alginates are the choice of encapsulating agents for this process. Cells along with coating agents are projected through a high pressure nozzle. However, this method is a sophisticated method and, thus, not easy to use.

Probiotic encapsulation has been successfully used to produce various food products such as yogurt (Weichselbaum, 2009), cheese (Gardiner et al., 1999), frozen dairy desserts (Ming-Ju & Kun-Nan, 2007), and other such dairy products. Other products including beverages, juices, and soft drinks are chosen for such incorporation of probiotic cells for their delivery to the consumers.

1.5 Recent advances on lactose intolerance

Lactose, the milk carbohydrate comprised of glucose and galactose connected by β-1,4 glycosidic linkage is found in most of the milk and milk products. Lactose is considered to be the most important source of energy for infants, providing almost half of the total energy requirement. Human milk contains an average of 7% lactose in comparison to bovine milk which contains 4.5% on average (Wijesinha-Bettoni & Burlingame, 2013). Apart from energy, lactose also promotes the absorption of minerals such as calcium, phosphate, manganese, and magnesium in human body (He, Priebe et al., 2008; He, Venema et al., 2008).

Lactose digestion occurs in the small intestine with the help of the enzyme lactase-phlorizin hydrolase, also known as β-D-galactosidase; β-D-galactoside galactohydrolase, EC 3.2.1.23, expressed on the villi of the epithelial cells (Silanikove et al., 2015). Lactase is responsible for the breakdown of lactose to its monomeric units which are then absorbed in the blood stream (Brown-Esters et al., 2012). While glucose is used up as an energy source, galactose helps the brain development in infants and biosynthesis of glycolipids and glycoproteins (Silanikove et al., 2015). The lactase activity is mostly lost after weaning in most of the mammals, and this loss is found to be genetically controlled (Brown-Esters et al., 2012; Suri et al., 2019). This lactase deficiency can result in a condition in which the person is not able to digest and absorb lactose, resulting in the problem called lactose intolerance. Still, in several people (∼25%) the lactase activity persists throughout life, which is also genetically controlled (He, Priebe et al., 2008; He, Venema et al., 2008; Swallow, 2003). Most commonly used terminologies are lactose malabsorption, lactase deficiency, and lactose intolerance. Lactase deficiency is almost always responsible for indigestion or malabsorption (Wilt et al., 2010). While lactose malabsorption occurs when undigested lactose, without getting absorbed, reaches the colon and undergoes fermentation by colonic microflora, increasing the osmotic load, and resulting in production of short chain fatty acids and gases such as carbon dioxide, methane, and hydrogen. The development of symptoms of gastrointestinal disorders such as gas, bloating, abdominal cramps, vomiting, nausea, and diarrhea after consuming large amount of lactose-containing food is termed as lactose intolerance (Szilagyi & Ishayek, 2018). Incidences of lactose intolerance in adults varies from 50% to 90% in African, Asian, and South American countries, while European and North American countries have 5%−15% (Misselwitz et al., 2013).

Lactose intolerance (LI) has been classified into three types. The most common is the primary LI, characterized by a continuous decrease in the lactase production. Secondary LI is associated with any illness or injury to the small intestine, resulting in less lactase expression. In young children the main causes of secondary LI are bacterial overgrowth, celiac disease, or Crohn's disease. Congenital LI also known as alactasia is a rare and severe autosomal recessive disorder of infants. In such cases the lactase activity is either completely absent or is present at very low levels. The patients are on a lactose-restricted diet throughout their lives (Savilahti et al., 1983).

Malabsorption is considered to be a precondition for lactose or food intolerance; however, the threshold for tolerance of dietary lactose varies from person to person (Suri et al., 2019), amount consumed, residual lactase activity (Swallow, 2003), transit time in gut (Zhao et al., 2010), colonic microflora, and the role of other dietary components consumed along with lactose (He, Priebe et al., 2008; He, Venema et al., 2008). The symptoms of lactose intolerance normally occur after 30 min to 2 h of consuming lactose containing food. Gastrointestinal symptoms after ingestion of milk or milk products may be misleading; therefore, diagnostic tests are more relied upon for the reasons of symptoms (Brown-Esters et al., 2012). The most widely used diagnostic test for lactose malabsorption is the hydrogen breath test (HBT), hydrogen being the gas produced by colonic microflora when they feed on undigested lactose (Casellas et al., 2009). Along with HBT other tests used are the lactose tolerance test for checking the increase in blood sugar level after lactose consumption; genetic test to test genetic-13910C/T polymorphism, and lactase activity at jejunal brush border to check the lactase activity in a biopsy sample (Maiuri et al., 1991).

1.5.1 Management of lactose intolerance

It has been reported by many authors that there exists a direct relation between amount consumed and severity of symptoms (Brown-Esters et al., 2012; Corgneau et al., 2017). Lactose in small doses of less than 12 g results in no symptoms, while amount between 20 and 50 g show considerable symptoms. The treatment of lactose intolerance includes the restriction on lactose-containing foods or taking it in reduced amounts. Depending upon the product composition, one glass of milk (250 mL) per day is tolerable to most of the lactose-intolerant patients. Numbers of dairy products are having less amount of lactose, owing to their processing techniques; in the cheese manufacturing process most of the lactose is moving in the whey portion. This is similar in yogurt where the lactose content is less, as most of it is converted to lactic acid by LAB (Corgneau et al., 2017). A 43 g of hard cheese contains less than 1 g of lactose; such

products can be taken in moderate amounts by lactose intolerant patients. It has been reported that the problem varies over time through adaptation of colon microflora to lactose (Brown-Esters et al., 2012).

It is to be noted that the removal of milk and milk products from the diet may result in development of micronutrient/mineral deficiencies. Milk and milk products are a major source of minerals and vitamins such as calcium, phosphorus, choline, vitamin B12, vitamin A, and riboflavin (Dekker et al., 2019). Barr (2013) in his survey found that the lactose intolerant patients intake less amounts of calcium (388−739 mg/day), which is below the Recommended Dietary Allowance (RDA) of 1000 mg/day. This lower intake was found to be associated with poor bone health, hypertension, and risk of diabetes mellitus (Pasin & Comerford, 2015).

In order to meet the dietary requirements of calcium and protein, the dairy industry has come up with lactose-free products prepared by an enzymatic hydrolysis technique using β-galactosidase enzyme that breaks down the lactose present in milk (Churakova et al., 2019). The resulting product is more susceptible to protein degradation as the lactose is replaced by glucose and galactose due to the action of enzymes that can react with the essential amino acid lysine at a higher rate than lactose itself to give Maillard browning products (Naranjo et al., 2013). Lactose hydrolyzed milk is found to be sweeter than regular milk because of monosaccharides, but in general, liked by the consumers the same as standard milk (Li et al., 2015).

The enzymes commercially used for the production of lactose-free dairy products are acid lactases and neutral lactases. While acid lactases are used as nutritional supplements and breakdown lactose in the stomach, neutral lactases are commercially used for production of lactose-free products. These enzymes can be extracted from microbial, plant, and animal origin for commercial applications, but the most common source used is microorganisms because of higher productivity thereby reducing the cost of the final product (Saqib et al., 2017). Most commonly used microbial sources are *Aspergillus* and *Kluyveromyces* (Zhou & Chen, 2001).

Enzyme immobilization is commonly used these days as a technique to enhance the enzyme stability and prevention of product contamination. Immobilization of β-galactosidase for commercial production of lactose-free or lactose-hydrolyzed milk is a promising technology for the food industry (Liu et al., 2012). Immobilization can prevent the enzyme denaturation while processing the food under different temperatures and pH conditions, thus, creating the possibility of reusing the enzyme a number of times. This can be achieved by different methods such as covalent attachment, adsorption, entrapment, encapsulation, and chemical aggregation. Wolf et al. (2018) immobilized β-galactosidase enzyme in gum arabic based hydrogel for production of lactose-free milk. They reported that the immobilized enzyme could work for more than three cycles of lactose hydrolysis without losing it activity while free enzyme could work only for one cycle.

Zhang and Zhong (2018) freeze dried solid/oil/water emulsion containing lactase to add lactase in milk products. Lactase powder was suspended in anhydrous milk/Span 80 and emulsified with lecithin and sodium caseinate (1:5). The presence of sodium caseinate preserved the lactase activity. The encapsulated lactase was released gradually during simulated digestions to break lactose more effectively than free lactase.

Liquid milk for lactose intolerant patient is usually ultra high temperature (UHT) sterilized and stored at room temperature up to 9 months. The product normally undergoes chemical changes during storage because of the presence of reducing sugars reactivity and also the side reactions of lactase enzymes in form of proteolysis and release of amino acids. Bottiroli et al. (2020) demonstrated that batch method is better that “in pack” system. In the process different lactase preparations were added to milk before thermal treatment. The chemical and sensory changes were studied up to 120 days at 20°C (68°F). Upon UHT processing the proteolytic effect is minimized. Color and sensory changes were not found to be poorly correlated to lactase. The study suggested that production costs can be reduced by crude lactases for the batch processes.

Suebsiri et al. (2019) checked the applicability of ohmic heating over conventional pasteurization and reported that the electrical conductivities of lactose-free milk ranges from 0.592 and 1.320 s/m, which indicates that the lactose-free milk can be efficiently processed by the ohmic heating process. Two different electrodes were used to check the metal contamination during processing. It was found that the milk processed using stainless steel electrodes contained high levels of metal contamination of chromium and iron, while the titanium electrode showed a safe level of chromium and no traces of iron.

Membrane processes such as ultrafiltration and microfiltration are also commercially used to produce low lactose or lactose-free milks. The process results in milk with only 1.6% of lactose content without any change in the taste. The milk can then be treated with enzymes to remove the lactose content balance (Corgneau et al., 2017).

In recent years, the use of probiotics as potential compensation for lactase deficiency has gained high interests. Probiotics are live microorganisms that replenish the healthy flora of GIT. These probiotics serve a dual purpose: it decreases the lactose content by fermentation and also supplies lactase enzymes which enter the small intestine with the

fermented products and help in colonic fermentation (He, Priebe et al., 2008; He, Venema et al., 2008). The most common probiotic bacteria are from the strains of *Bifidobacterium* and *Lactobacillus* as these are the predominant strains of gastrointestinal microflora (Surendran Nair et al., 2017). Lactose present in yogurt and similar probiotic products are digested in a better manner than normal milk by lactose intolerant subjects. Fermentation results in utilization of 25%–50% of lactose, decreases the content to approximately 4% (Brown-Esters et al., 2012). In Western countries, 50% of the world yogurt market is dominated by many international manufacturers of cultured milk and milk blends (Silanikove et al., 2015). The effect of different probiotics when administered to lactose intolerant patients has been summarized in Table 1.1.

Frozen yogurt is gaining popularity among consumers over ice cream because of its low fat content as well as the presence of healthy viable microorganisms. Skryplonek et al. (2019) investigated the effect of k-carrageenan and corn starch on various properties of lactose-free frozen yogurt. In the study, simultaneous fermentation with yogurt culture and enzymatic hydrolysis of lactose using Ha-lactase enzyme was carried out to lower the lactose concentration below the detection limit. They were able to produce lactose-free frozen yogurt using 0.15% k-carrageenan which showed a positive effect on sensory properties and also reduced the coarse texture, thus, showing a potential for commercialization of lactose-free frozen yogurt.

Tolerance of lactose can also be improved by consuming milk and milk products with other meals rather than alone; it improves lactose indigestion and minimizes the symptoms due to slowing down the transit time of lactose through the GIT because of the presence of additional foods (Dehkordi et al., 1995).

As no specific guidelines for managing LI are available, the most common approach is to restrict dairy products from the diet (Montalto et al., 2006), but this approach may result in other micronutrient deficiencies so alternative strategies need to be considered.

1.6 Exopolysaccharides from fermented dairy products

LAB are a group of seven genera namely *Enterococcus, Streptococcus, Lactobacillus, Lactococcus, Oenococcus, Leconostoc* and *Pediococcus*. These have a long history of safe use in different fermented food products and are generally recognized as safe (GRAS) with many having the qualified presumption of safety (QPS) status (Saadat et al., 2019). Also the foods produced by the LAB bacteria are given a GRAS status. LAB are commonly found in most fermented foods such as yogurt, cheese, wine, sausage, sour dough sauerkraut, and many more (Xu et al., 2019). Besides their significant role in production of fermented foods they also synthesize many other metabolites such as bacteriocins, organic acids, aromatic compounds, fatty acids, and EPSs (Oleksy & Klewicka, 2018). EPSs are long-chain high molecular weight biopolymers, linear or branched, composed of sugar units or sugar derivatives joined by glycosidic linkages (Liu et al., 2011). These can be classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS), based on chemical composition and their synthesis mechanism (Zununi Vahed et al., 2017). In general, homopolysaccharides comprised of a single monomer unit, glucose or fructose, are produced externally to the cell by an enzyme, glycanosucrase or fructansucrase, secreted by the bacteria, from sucrose or starch. Generally, the molecular weight of homopolysaccharides is higher than the heteropolysaccharides (Caggianiello et al., 2016). For example, the molecular weight of homopolysaccharide D-glucan produced by *L. reuteri* was 4.3×10^7 Da while molecular weight of heteropolysaccharides range from 10^4 to 10^6 Da (Miao et al., 2014). Glucose contacting HoPS include α- and β-glucans such as dextran; whereas fructose containing HoPS include fructans such as levan (Xu et al., 2019). HoPS-forming LAB find applications mostly in cereal products that contain sucrose as an ingredient.

In HEPS the biosynthetic pathway is more complex and not fully understood. It is recognized that it involves the transport of sugar into the cytoplasm where the repeating units are synthesized; whereas, polymerization takes place on the outside of the cell. HePS are mainly composed of repeating units ranging from disaccharides to ocatasaccharides with mainly galactose, glucose, and rhamnose present in different ratios. These are usually branched and have a charge which affects its interaction with other food components, which is crucial for food applications. HePS finds application mainly in dairy products containing lactose as a substrate for EPS production (Loeffler et al., 2020).

1.6.1 Functional properties

With the advent of functional food markets and a rapid increase in the demand of probiotic-containing foods because of its health and economic benefits, EPS producing strains have been studied for their role in probiotic colonization. EPS form a protective covering around the EPS-producing strains and improves their tolerance to the GIT environment thereby improving the chance of survival. EPSs can also promote the probiotics colonization in the intestines through

TABLE 1.1 Effect of probiotics in lactose intolerant patients

Sr. no.	Probiotic strain	Strain specification	Strain predicted benefit	Influence on lactose intolerant patients	References
1.	*Bifidobacterium longum*	Gram positive, nonmotile, nonsporulating lactic-acid fermenting bacteria	Antiallergy effects Reduces harmful bacterial strains Improves gut health by increasing the defecation frequency and stool characteristics shows antiinflammatory effect offers protection from toxins maturation of immune cells for proper functioning	No change in the degree of digestion of lactose in the gut but reduces the symptoms after its supplementation	He, Priebe et al. (2008), He, Venema et al. (2008), Zhong et al. (2003)
2.	*Bifidobacterium animalis*	Gram positive, anaerobic, rod shaped bacteria	Decreases the serum cholesterol level Prevents colorectal cancer Improves gut health, that is, reduces constipation, gut inflammation and increases gut transit time	Improves the digestion of lactose sugar and reduces symptoms of lactose intolerance (diarrhea and regulation of gut microbiota)	Luyer et al. (2010), Zhong et al. (2006)
3.	*Lactobacillus bulgaricus*	Gram positive facultative anaerobe, nonmotile lactic acid bacteria	Reduction of intestinal infections through changing pH of the gut by release of acids. Production of natural antibiotics and blockage of adhesion sites in the mucosal layer of the intestines	Improves lactose digestion and eliminates lactose intolerance symptoms	Luyer et al. (2010), Dolores, & Martínez, (2007)
4.	*Lactobacillus reuteri*	Gram positive, rod-shaped anaerobic strain colonizing the gut	Protection from harmful bacteria Stabilizing the permeability of the intestines. Decreases the symptoms of nausea, flatulence and diarrhea. Rises the secretion of insulin and the strain shows high beta-galactosidase activity	Shows a reduction of symptoms of lactose intolerance especially diarrhea among children	Dolores, & Martínez, (2007)
5.	*Lactobacillus acidophilus*	Gram positive bacteria that naturally occurs in the gut	Controlling intestinal infections. Controlling serum cholesterol amount. Has anticarcinogenic activities	Limited to no improvement in digestion of lactose among the lactose intolerant patients	Wilt et al. (2010)
6.	*Lactobacillus rhamnosus*	Gram positive lactic acid fermenting bacteria	Improving immune responses. Antiinflammatory	Improve lactose digestion and reduce the lactose intolerance symptoms (diarrhea and regulation of bowel movements). Encourages growth of other microbes that aid in the digestion of lactose	Agustina et al. (2007)
7.	*Saccharomyces boulardii*	Acid-resistant, tolerates high temperature, nonpathogenic tropical yeast species	Rises the functioning of the intestinal activities (disaccharides, α-glucosidases, alkaline phosphatases and amino peptidases). Protection against harmful microbes	Shows relief from lactose intolerance symptoms, diarrhea, especially among children and enhances their weight regain	Luyer et al. (2010)
8.	*Streptococcus thermophilus*	It is a gram-positive lactic acid fermenting, facultative anaerobic bacteria	Improves carbohydrate metabolism	Does not have any improvements on lactose intolerance symptoms	Ojetti et al., (2009)

immune evasion by forming a defensive shield (Caggianiello et al., 2016). Subsequently, pathogen settlement can be inhibited by creating a competition for nutrient and space by EPS-producing strains. EPSs also show antibacterial activity thus preventing the pathogen infection.

Many EPS have been investigated for their prebiotic potential. In general, HoPS have been demonstrated to have prebiotic potential while HePS have shown a immunomodulatory effect. Russo et al. (2012) reported the production of β-glucan by strains *L. planatarum* WCFS 1, *L. planatarum* WCFS 1b-gal, and *L. acidophilus* NCFM. *Weissella cibaria* RBA12 produced dextran showed high tolerance to artificial gastric juice, α-amylase and intestinal juices and improved the growth of probiotic *Bifidobacteria* and *Lactobacillus* spp. Kefiran which is a branched hydrosoluble glucogalacta produced by *Lactobacillus kefiranofaciens* enhanced the growth of *Bifidobacterium* but no effect was observed on *Lactobacillus* (Hamet et al., 2016).

EPSs possess different potential health benefits including immunomodulatory properties, anticancer, antioxidant activity (Abid et al., 2018), antibiofilm agents to prevent pathogenic bacteria adhesion, blood glucose (Oleksy & Klewicka, 2018)/cholesterol lowering properties (Korcz et al., 2018), and antihypertensive activity (Harutoshi, 2013).

1.6.2 Food applications

EPS has gained importance because of its potential application in fermented dairy products. It has been known to perform various roles such as improvement in texture, sensory properties, dietary fibers, coating agents, as well as it inhibit syneresis even at low concentrations. EPS solubilize in water and interact with water molecules to form gel thereby affecting the rheological properties and provide physical stability to foods (Xu et al., 2019). The EPS produced by LAB and *Bifidobacterium* can improve the water holding capacity and viscosity of yogurt. In addition, it can increase the yield of cheese by increasing water and fat retention giving a creamier and softer texture. *Streptococcus thermophilus* EPS can replace stabilizer used in ice cream and give the desirable viscosity and pseudoplastic fluid behavior (Delattre et al., 2016).

Commercially development of a set yogurt texture can be a problem because of the application of mechanical devices. These devices may result in structural damage to the coagulum at different stages of manufacture (Laws & Marshall, 2001). In general, the concentration of EPS plays an important role in gelation process of yogurt. This resulted in selecting strains of LAB producing higher concentration of EPS for yogurt production. Still, many reports that give contradictory results showed that monosaccharide composition, molar mass, degree of branching, and interaction of EPS with milk components play vital role in textural properties (Gentès et al., 2011).

It has been reported that EPS forming and nonforming strains may produce the acid milk gels with different rheological properties. Khanal and Lucey (2017) studied the EPS produced by two strains of *S. thermophiles* for yield, molar mass, and rheological properties of gels formed during fermentation. They reported that while St-143 fermented milk showed weaker gel storage modulus of 26 Pa, ST-10255y showed a stiffer gel with storage modulus value of 82 Pa. The observed difference in properties could be due to the difference in molar mass and chemical structure of EPS produced during fermentation. Surber et al. (2019) classified 20 *S. thermophilus* strains on the basis of EPS concentration (8−126 mg GE/kg) and ropiness (thread length: 15−80 mm). Acidification and gelation results showed that fermentation time and gel stiffness was higher for strains that produced ropy EPS. An additional improvement in gel stiffness was observed for the strains that also produced cell-bound EPS, which emphasizes the significance of both ropy and cell-bound EPS for refining acid gel properties.

The role of EPSs in improvement of viscosity and water holding capacity are ascribed to its inherent properties as well as its interaction with the proteins of the food (Tidona et al., 2016), which in turn are dependent on EPS structure, its molecular weight, and side groups (Laws, Gu, & Marshall, 2001; Xu et al., 2019). Gentès et al. (2016) studied the impact of three EPS producing strains with known structures on apparent viscosity. The three strains selected were *S. thermophilus* producing anionic, stiff, and linear EPS; *L. delbrueckii* subsp. *bulgaricus* LB1 with neutral, stiff, and ramified EPS; and *L. delbrueckii* subsp. *bulgaricus* LB2 with neutral, flexible, and highly ramified EPS. The results showed that higher viscosity values were observed with stiff, linear, and slightly branched EPS from *S. thermophilus* and LB2.

Zhang et al. (2017) produced ice cream using an EPS producing strain of *L. planatarum* that produced ropy EPS up to 4.84 mg/g. The production of EPS along with a change in pH increased the viscosity of ice cream up to 131 mPa.s, overrun and melting resistance was also increased and fat destabilization decreased.

Apart from the wide applications of EPS in the dairy industry, EPS has also found applications in the bakery industry owing to its physiochemical properties similar to that of hydrocolloids or gums. Thus, the properties of EPS and its in situ production make it a natural alternative of hydrocolloids for gluten and gluten-free products (Xu et al., 2019). The influence of EPS formed during sourdough fermentation on the quality parameters of bread and steamed breads

were compared by Xu et al. (2020). EPS producing strains used for sourdough fermentation were *Fructilactobacillus sanfranciscensis, Weissella cibaria*, and *Leuconostoc mesenteroides*. Production of EPS enhanced the specific loaf volume, improved the crumb texture, and retarded the bread staling. The effect on steamed bread was more distinct than normal bread in terms of quality. Galle et al. (2010) observed an increase up to 35% wheat bread volume rise when added with 20% of *W. cibaria* fermented sour dough. Similar results have been reported for gluten free sourdoughs of buckwheat, quinoa and teff (Wolter et al., 2014) and buckwheat and rice flour (Rühmkorf et al., 2012).

In the meat industry, EPS can be used to replace hydrocolloids and phosphates owing to its water holding capacity and improvement in textural properties (Yilmaz et al., 2015). EPS-producing LAB strains can be used in raw fermented sausages (spreadable). The additional advantage of an in situ EPS production is its effect on sensory properties and giving the desired spreadability even at reduced fat content (Amini Sarteshnizi et al., 2015). In order to replace the phosphates from cooked ham Hilbig et al. (2019) checked the ability of different strains for EPS production in cooked ham model system. They reported that the selected strains *Lactobacillus sakei* TMW 1.411 (HoPS) or *L. planatarum* TMW 1.1478 (HePS) were not able to multiply under the cooked ham model but did produced EPS although in less concentration without changing the pH of the system. This was because of the combined effect of cold temperature and salt stress which did not allow the strains to grow.

EPS produced by LAB have diverse applications in the food industry with more effort still underway to exploit its potential in other sectors. However, more work is needed in exploring prominent strains and optimizing the process parameters to decrease the cost and improve the yield of EPS. In order to improve its application potential, it is important to develop a correlation between structure, properties, and health benefits.

1.7 Conclusion and future prospective

Fermented dairy foods have been consumed worldwide for their wide benefits. Functional foods containing live microorganisms and bioactive products made thereof are considered as the newest trend in the food market. Techniques like microencapsulation are used to improve the viability of the live cells that are incorporated into these functional foods. These fermented products are a boon to conditions like lactose intolerance. Various food applications have been designed using functional properties like production of EPSs for development of various functional dairy food products. Future prospects hold bright for fermented dairy foods.

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Chapter 2

Recent advances in microbial diversity usage in fermented dairy microbial products

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2.1 Introduction

For thousands of year dairy products have been the major parts of human diet because fermented milk products like curd, cheese, and butter could be better preserved than the fresh raw milk from which they were made (Kok & Hutkins, 2018). Most fermented dairy products naturally contain organic acids, ethanol, or other microbial compounds that inhibit the growth of the spoilage organisms and food borne pathogens (Ross & Hill, 2002). In our daily life, fermented dairy products play an important role. These products contain a diverse microbiota (Fernandez et al., 2015) apart their preservation qualities. Fermented foods have other attributes like flavor, texture, and appearance, and also add functional and economic value. Fermented foods are the most important sources of nutrients in many parts of the world (Marsh et al., 2014). Bread and sausage are the cultured dairy products, they are very rich in protein, vitamins, and minerals. Fermentation processes also reduce the amount of lactose and fermentable sugars and increase phenolic compounds which provide antioxidant activity (Shah, 2006).

2.2 Global trends and consumption patterns of milk products

Nowadays, consumers prefer foods that promote good health and prevent disease (Salminen et al., 1998). Furthermore, these foods must fit into current lifestyles providing convenience of use, good flavor, and an acceptable price value ratio. Such foods constitute current and future waves in the evolution of the food development cycle. There are several principal reasons for the success of fermented dairy products, which relate to nutrition and health, versatility and marketing. Scientific and clinical evidence is also mounting to corroborate the consumer perception of health from fermented milks. The increasing demand from consumers for dairy products with "functional" properties is a key factor driving value sales growth in developed markets (Chandan, 1999). This led to the promotion of added-value products such as probiotic and other functional yogurts, reduced-fat and enriched milk products, and fermented dairy drinks and organic cheese. The manufacture of cultured dairy products represents the second most important fermentation industry (after the production of alcoholic drinks) and grew at six times the rate of total dairy growth between 1998 and 2003 in value terms, according to a recent market survey (Adwan, 2003; Anon, 2003). The fermented milk products are significant to the Indian dairy industry as they act as an outlet for dairy plant milk surplus and are seeking big markets abroad (Rajorhia, 1998). Also they have tremendous traditional appeal throughout Indian masses, especially the affluent consumer groups with regional preferences like shrikhand and misti doi becoming very popular. The Indian fermented milk products mainly include dahi, lassi, shrikhand, buttermilk, and misti doi. Milk occupies an exalted position in India (Gupta, 2000). Its roots go back to some 6000 years when milk animals were domesticated. Simple processes were developed to preserve milk's nutritive goodness as a means to protect and promote health. The process of food fermentations was probably known to people inhabiting India in the Paleolithic and Neolithic times judging from the records of food habits. A wide range of sweets were produced for consumption on festive occasions (Gupta, 2000). They included rasogolla, sandesh, burfi, peda, shrikhand, gulabjamun, lassi, misti doi, and kheer (rice pudding), combining

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delicious taste and flavor with fitness and health. These ethnic products constitute the world of traditional dairy products (Aneja et al., 2001). India is the largest producer of milk in the world with over 50 million women and 15 million men involved in dairy enterprises. India contributes around 13% to global milk production with 70 million milk producers owning 90 million milking animals (Anon, 2005a). The estimated production of milk in India in 2004–05 was 91 million tons, projected to increase to 127 million tons by the end of the 11th five year plan (Nagpal et al., 2012). The dairy products are consumed not only for meeting the nutritional requirements of the consumers, but also for their role in preventing various disorders such as obesity (Jaffiol, 2008), osteoporosis (Uenishi, 2006), dental caries (Ferrazzano et al., 2008; Shimazaki et al., 2008), poor gastrointestinal health (Pufulete, 2008), cardiovascular disease (Lamarghe, 2008), hypertension (Jauhiainen & Korpela, 2007), colorectal cancer (Weaver, 2009), bone ailments, ageing (Ginter, 2008), and others (Sharma & Rajput, 2006; Fig. 2.1).

2.3 History of fermented dairy products

The dairy product "dahi," is a yogurt-like fermented milk product of India. It was mentioned in about 6000–4000 BCE in the Rig Veda and Upanishad, ancient sacred books of the Hindus (Yegna Narayan Aiyar, 1953). Likewise, the Turkish people in Asia also made a similar product, giving it the name "yoghurut" (Rasic & Kurmann, 1978). Evidence of production and consumption of cheese in Kujawy of Poland was recorded around 5500 BCE (Salque et al., 2012).

2.4 Fermentation

Fermentation is traditionally one of the oldest and cost-effective methods for producing and preservation. It has been practiced since ancient times (Jeyaram et al., 2009). Preparation of traditional fermented food is one of the oldest biotechnological processes around the world (Sekar & Mariappan, 2007). There are two ways for prepare fermented food: one is natural fermentation, the second is by adding starter culture comprised of efficient microorganisms that transform the substrates into edible products are ethnically and socially conventional to the local people (Angmo et al., 2016). Nowadays, more than 5000 various types of fermented foods are consumed by diversified people living worldwide (Ray et al., 2016). The makeup of fermented food is an important contribution to our diets since time immemorial because it is a cost-effective technology that preserves food, improves nutritional value, and enhances its sensory qualities (Marco et al., 2017). Microorganisms play an important role in the process of fermentation. The most commonly found microorganisms are lactic acid bacteria (LAB), accomplishing a crucial role in the preservation and production of

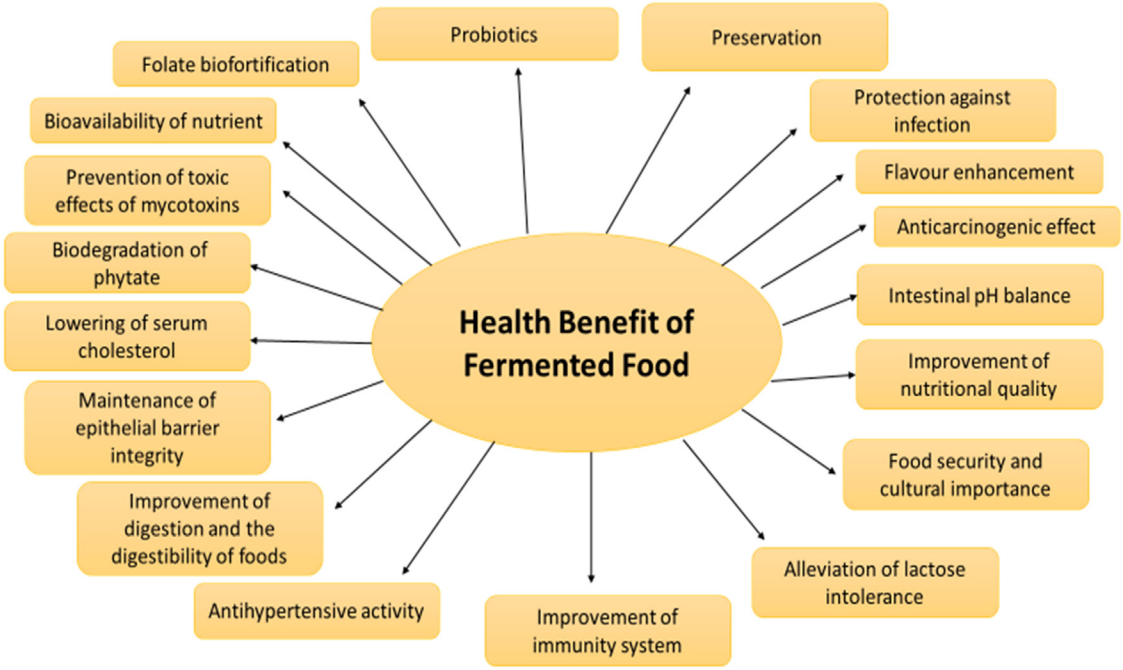

FIGURE 2.1 Benefits of fermented food.

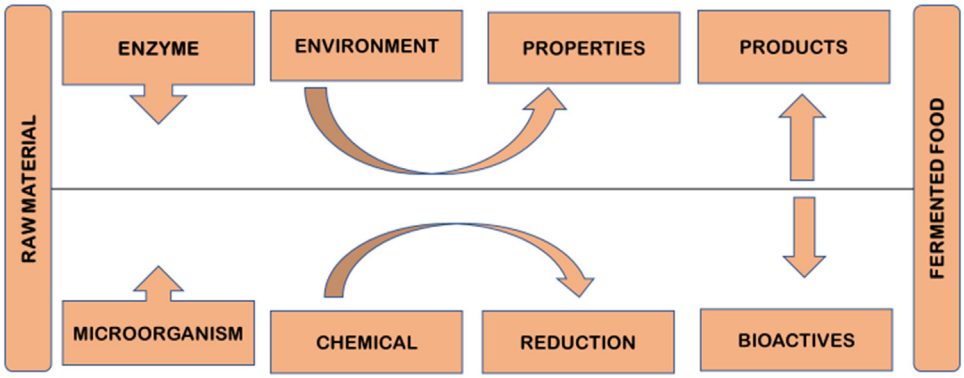

FIGURE 2.2 Product formation in fermentation.

nutritious fermented foods (Satish et al., 2013). Many bacteria associated with fermented foods produce antimicrobial bioactive molecules, such as hydrogen peroxide, organic acids, and bacteriocins, that make them effective biopreservatives (Ananou et al., 2007; O'Sullivan et al., 2010) and produce nutraceuticals to create functional foods with increased bioavailability of nutrients (Angmo et al., 2016; Divya et al., 2012; Toma & Pokrotnieks, 2006; Fig. 2.2). Fermentation enhances the absorption of vitamins and minerals. Calcium, phosphorous, and iron are better utilized by consuming fermented milks. The reason could be the release of phospho-peptides by hydrolysis of casein which promotes absorption. Fermentation also reduces the lactose content of milk which is beneficial for the lactose intolerant population. Khetra et al. (2011) reported that nutritional functions of fermented milks involve a supply of macronutrients including carbohydrate, fat, and protein in easily digestible forms and micronutrients including calcium, phosphorous, magnesium, and zinc, and certain water soluble vitamins.

2.4.1 Fermentation process

2.4.1.1 Mechanism of gel formation

During the process of fermentation in milk, a gradual conversion of lactose into lactic acid by suitable microorganisms (Amice-Quemeneur et al., 1995) causes the pH to decrease its impacts on the properties of casein, which influences their gelation properties during the formation of cultured products (Singh et al., 1997). Casein micelles are composed of different protein fractions that are associated with one another via Ca-phosphate bridges (Tamime & Robinson, 1999). Acid production by LAB results in several changes in the physicochemical properties (like reduction in the surface charge of casein micelles) (Lucey, 2004; Lucey & Singh, 2003).

2.4.1.2 Physicochemical changes

During the process of milk fermentation, LAB in the culture hydrolyze the lactose into lactic acid as a major product and increase acidity by 90% (Mathur, 1991). In this process, lactic acid combines with calcium to form calcium lactate. The casein devoid of calcium is coagulated (Sindhu et al., 2000). Finally, milk is converted into a jelly-like structure; principally, the protein and syneresis is manifested by the appearance of a thin exudate of clear whey on the surface of the product. After fermentation of milk, nitrogen and ammonia nitrogen increase and protein nitrogen decreases while total nitrogen remains the same.

2.5 Classification of fermented milk

Fermented milks are manufactured throughout the world and approximately 400 generic names are applied to traditional and industrialized products (Khurana and Kanawji, 2007; Kurmann et al., 1992). In the 1980s, Kurmann (1984) classified fermented milks into a "family tree" (Bylund, 1995), which was based primarily on the optimum growth requirements of the starter cultures (i.e., mesophilic and thermophilic microflora) (Tamime & Marshall, 1997; Tamime & Robinson, 1999). Robinson and Tamime (1995) proposed a scheme for the classification of fermented milks into lactic acid by different type of microorganism (Fig. 2.3). Fermented milks (Table 2.1) are classified into two major groups based on the presence of dominant microorganisms: (1) lactic fermentations which are dominated by species of LAB, and consist of the thermophilic type (e.g., yogurt, Bulgarian buttermilk), probiotic type (acidophilus milk, yakult, bifidus milk), and the mesophilic type (e.g., natural fermented milk, cultured milk, cultured cream, cultured buttermilk);

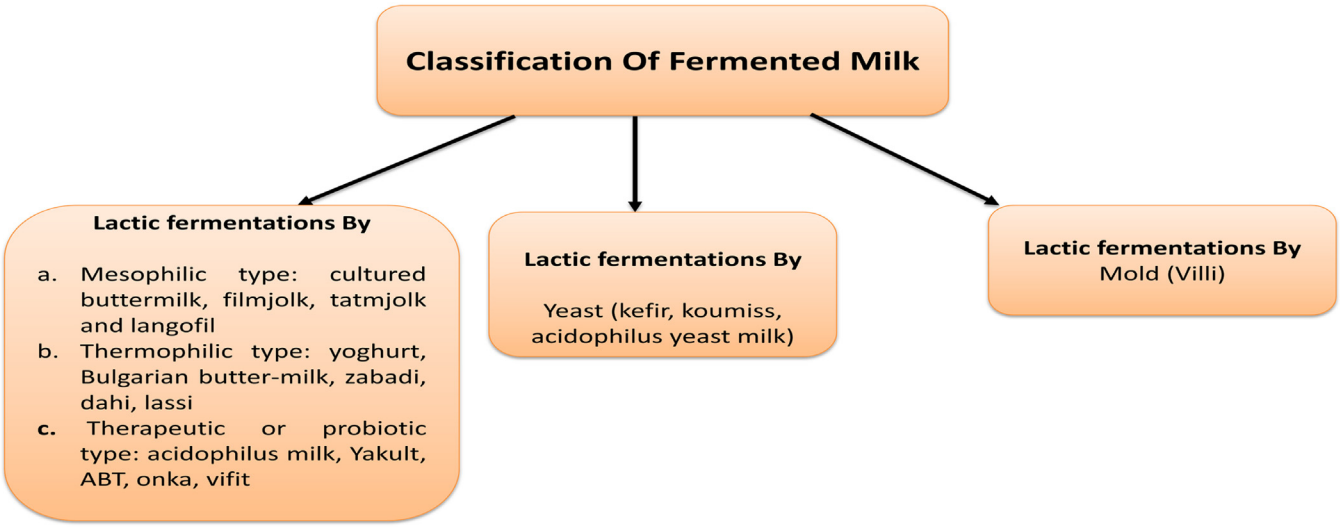

FIGURE 2.3 Classification of fermented milks into lactic acid by different type of microorganism.

(2) Fungal-lactic fermentations where LAB and yeasts species cooperate to generate the final product that consists of alcoholic milks (e.g., kefir, koumiss, acidophilus-yeast milk), and moldy milks (e.g., viili) (Mayo et al., 2010). Starter cultures in milk fermentation are of two types depending on the principal function. First is primary cultures to participate in the acidification, like *Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis, Lactobacillus delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *lactis, Lactobacillus helveticus, Leuconostoc* spp., and *Streptococcus thermophilus* (Parente & Cogan, 2004) and secondary cultures increase flavor, aroma, and maturing activities (Topisirovic et al., 2006). Secondary cultures used in cheese making are *Brevibacterium linens, Propionibacterium freudenreichii, Debaryomyces hansenii, Geotrichum candidum, Penicillium camemberti*, and *Penicillium roqueforti* (Coppola et al., 2006; Quigley et al., 2011). Some nonstarter lactic acid bacteria (NSLAB) microbiota is usually present in high numbers which include *Enterococcus durans, Enterococcus faecium, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus salivarius*, and *Staphylococcus* spp. (Briggiler-Marcó et al., 2007; Fig. 2.4).

Yogurt is a widely consumed highly nutritious fermented milk as a coagulated milk product resulting from the fermentation of milk by *S. thermophilus, L. delbrueckii* subsp. *bulgaricus* (formerly *Lactobacillus bulgaricus*) *Lactobacillus acidophilus, Lb. casei, Lactobacillus rhamnosus, Lactobacillus gasseri, Lactobacillus johnsonii*, and *Bifidobacterium* spp., are among the most common adjunct cultures in yogurt fermentation (Guarner et al., 2005; Tamime & Robinson, 2007). Fermented milk products that are manufactured using starter cultures containing yeasts include acidophilus-yeast milk, kefir, koumiss, and viili. *L. acidophilus, Lactobacillus amylovorus, Lactobacillus crispatus, Lactobacillus gallinarum, L. gasseri*, and *L. johnsonii* are reported from acidophilus milk (Berger et al., 2007; de Ramesh et al., 2006). Natural fermented milks are one of the oldest methods of milk fermentation using raw or boiled milk to ferment spontaneously or by using the back-slopping method (Josephsen & Jespersen, 2004; Robinson & Tamime, 2006). In India, Nepal, Pakistan, Bhutan, and Bangladesh different types of naturally fermented milks like dahi, lassi, misti dahi, srikhand, chhu, chhurpi, mohi, philu, shoyu, somar (cow/buffalo/yak milk) (Harun-ur-Rashid et al., 2007; Patil et al., 2010; Sarkar et al., 2006; Tamang, 2010a; Tamang et al., 2012), kurut of China (Sun et al., 2010), aaruul, airag, byasulag, chigee, eezgii, tarag, and khoormog of Mongolia (Oki et al., 2014; Takeda et al., 2011; Watanabe & Wakasugi, 2008), ergo of Ethiopia, kad, lben, laban, rayeb, zabady, zeer of Morocco and Northern African and Middle East countries, rob (from camel milk), biruni (cow/camel milk), mish (cow/camel milk) of Sudan, amasi (hodzeko, mukaka wakakora) of Zimbabwe, nunu (from raw cow milk) of Ghana (Akabanda et al., 2013), filmjölk and långfil of Sweden (Mayo et al., 2010), koumiss or kumis or kumys or kymys of the Caucasian area (Wu et al., 2009).

2.6 Role of microorganism in milk fermentation technology

Microorganisms determine the characteristics of fermented food, for example, acidity, flavor and texture, as well as the health benefits that go beyond simple nutrition (Vogel et al., 2011). Microorganisms may be present as the indigenous microbiota of the food or as a result of the intentional addition of microorganisms as starter cultures in an industrial

TABLE 2.1 Classification of fermented milk.

S. No.	Product	Substrate	Sensory property and nature	Microorganism	References
1	Acidophilus milk	Cow milk	Acidic, sour, drink	Species of *Lactobacillus, Lactococcus*	Mayo et al. (2010)
2	Biruni	Cow/camel milk	Acidic, semi-liquid, drink	LAB	Jung (2012)
3	Chhu	Yak/cow milk	Cheese like product, curry, soup	*Lactobacillus farciminis, Lactobacillus brevis, Lactobacillus alimentarius, L. salivarius, Lactococcus lactis, Saccharomycopsis* sp., *Candida* sp.	Dewan. and Tamang (2006)
4	Airag	Mare or camel milk	Acidic, sour, mild alcoholic, drink	*Lactobacillus helveticus, Lactobacillus kefiranofaciens, Bifidobacterium mongoliense, Kluyveromyces marxianus*	Watanabe and Wakasugi (2008), Watanabe et al. (2009b)
5	Ayib	Goat milk		*Canida* sp., *Saccharomyces* sp., *Lactobacillus* sp., *Leuconostoc* sp.	Odunfa and Oyewole (1998)
6	Butter	Animal milk	Soft paste, butter	LAB	Mayo et al. (2010)
7	Buttermilk	Animal milk	Acid fermented buttermilk	*Lactobacillus bulgaricus*	Mayo et al. (2010)
8	Cheese	Animal milk	Soft or hard, solid; side dish, salad	*Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis, Lactobacillus delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *lactis, L. helveticus, Lb. casei, Lactobacillus plantarum, L. salivarius, Leuconostoc* spp., *Streptococcus thermophilus, Enterococcus durans, Enterococcus faecium*, and *Staphylococcus* spp., *Brevibacterium linens, Propionibacterium freudenreichii, Debaryomyces hansenii, Geotrichum candidum, Penicillium camemberti, Penicillium roqueforti*	Parente & Cogan, 2004, Quigley et al. (2011)
9	Chhurpi (hard)	Yak/cow milk	Chewable milk, masticator	*L. farciminis, Lb. casei, Lactobacillus biofermentans, Weissella confusus*	Tamang (2010a)
10	Chhurpi (soft)	Yak/cow milk	Cheese-like product, soup, curry, pickle	*L. farciminis, Lactobacillus paracasei, L. biofermentans, L. plantarum, Lactobacillus curvatus, Lactobacillus fermentum, L. alimentarius, Lactobacillus kefir, Lactobacillus hilgardii, W. confusus, E. faecium, Leuconostoc mesenteroides*	Tamang et al. (2000)
11	Dahi	Cow/buffalo milk, starter culture	Curd, savory	*Lactobacillus bifermentans, L. alimentarius, L. paracasei, L. lactis, Streptococcus cremoris, Strep. lactis, S. thermophilus, L. bulgaricus, Lactobacillus acidophilus, L. helveticus, Lactobacillus cremoris, Ped. pentosaceous, P. acidilactici, Weissella cibara, Weissella paramesenteroides, L. fermentum, L. delbrueckii* subsp. *indicus, Saccharomycopsis* sp., *Candida* sp.	Harun-ur-Rashid et al. (2007), Patil et al. (2010)
12	Ergo	Milk	Acid fermented buttermilk	*Lactobacillus* sp., *Lactococcus* sp.	Steinkraus, 1997
13	Filmjolk	Cow milk	Less-sour than yoghurt, yoghurt-like	*L. lactis* and *L. mesenteroides*	Kosikowski and Mistry (1997)

(*Continued*)

TABLE 2.1 (Continued)

S. No.	Product	Substrate	Sensory property and nature	Microorganism	References
14	Gariss	Camel milk	Acidic, liquid, refreshing beverage	LAB	Akabanda et al. (2013)
15	Gheu/ghee	Cow milk	Soft, oily mass, solid, butter	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris*	Tamang (2010a)
16	Kefir	Goat, sheep, cow	Alcoholic fermented milk, effervescent milk	Torula *holmii,Torulaspora delbruechii, L. brevis, Lactobacillus caucasicus, S. thermophilus, L. bulgaricus, L. plantarum, Lb. casei, L. brevis*	Bernardeau et al. (2006)
17	Kesong Puti, Keso, kesiyo	Carabao's (buffalo) milk or cow carabao's milk, salt, Abomasal extracts coagulant, starter	White cheese, soft cheese	*L. helveticus, L. lactis, Lactobacillus rhamnosus, L. mesenteroides, L. acidophilus, L. plantarum, L. brevis, L. curvatus*	Kisworo (2003)
18	Kishk	Milk, wheat	Fermented milk wheat mix, drink	*L. plantarum, L. brevis, L. bulgaricus, Lb. casei, S. thermophilus*	Bernardeau et al. (2006)
19	Kurut	Yak milk	Naturally fermented milk, drink	LAB	Sun et al. (2010)
20	Kushuk	Milk, wheat	Fermented milk wheat mix, drink	*L. plantarum, L. brevis*	Bernardeau et al. (2006)
21	Koumiss	Milk	Acid fermented milk, drink	*L. bulgaricus, Torula* sp., *L. salivarius, Lactobacillus buchneri, Lactobacillus heveticus, L. plantarum, L. acidophilus*	Hao et al. (2010)
22	Laban rayed	Milk	Acid fermented milk, yogurt-like	*Lb. casei, L. plantarum, L. brevis, L. lactis, Sacharomyces kefir, Leuconostoc* sp.	Bernardeau et al. (2006)
23	Laban zeer	Milk	Acid fermented milk	*Lb. casei, L. plantarum, L. brevis, L. lactis*	Bernardeau et al. (2006)
24	Lassi	Cow Milk	Acidic, buttermilk, refreshing beverage	*L. Acidophilus, S. thermophilus*	Patidar and Prajapati (1998)
25	Långfil	Cow Milk	Elastic texture, sour, yogurt-like	LAB	Tamime (2005)
26	Leben/Lben	Cow Milk	Sour milk	*Candida* sp., *Saccharomyces* sp., *Lactobacillus* sp., *Leuconostoc* sp.,	Odunfa and Oyewole (1998)
27	Liban-argeel	Sheep, goat, cow, buffalo milk	Acid fermented milk	LAB	Bernardeau et al. (2006)
28	Maa	Yak milk	Mild-acidic, viscous, butter	LAB, yeasts	Tamang (2010a)
29	Maziwa lala	Milk	Yoghurt-like	*Strep. lactis, S. thermophilus*	Olasupo et al. (2010)
30	Mohi	Cow milk	Acidic, buttermilk, refreshing beverage	*L. alimentarius, L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris; Saccharomycopsis* spp. and *Candida* spp.	Dewan and Tamang (2007)

31	Mish	Cow/camel milk	Acidic, semi-liquid, refreshing beverage	LAB	Bernardeau et al. (2006)
32	Misti dahi (mishti doi, lal dahi, payodhi)	Buffalo/cow milk	Mild-acidic, thick-gel, sweetened curd, savory	*Streptococcus salivarius* subsp. *thermophilus, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. lactis* subsp. *lactis, Saccharomyces cerevisiae*	Ghosh and Rajorhia (1990), Gupta et al. (2000)
33	Nunu	Raw cow milk	Naturally fermented milk	*L. fermentum, L. plantarum, L. helveticus, L. mesenteroides, E. faecium, Enterococcus italicus, W. confusus; Candida parapsilosis, Candida rugosa, Candida tropicalis, Galactomyces geotrichum, Pichia kudriavzevii, S. cerevisiae*	Akabanda et al. (2013)
34	Paneer	Buffalo or cow milk	Whey, soft, cheese- like product, fried snacks, curry	LAB	Tamang (2012b)
35	Phrung	Yak milk	Mild-acidic, hard-mass like chhurpi, masticator	Unknown	Tamang (2012b)
36	Philu	Cow or yak milk, bamboo vessels	Cream like product, curry	*L. paracasei, L. bifermentans, E. faecium*	Dewan and Tamang (2007)
37	Pheuja or suja	Tea-yak butter, salt	Salty with buttery flavor, liquid, Refreshing tea	Unknown	Tamang (2010a)
38	Rob	Cow, goat, sheep milk	Mild-acidic, savory	LAB	Akabanda et al. (2013)
39	Shrikhand	Cow, buffalo milk	Acidic, concentrated sweetened viscous, savory	*L. lactis* subsp. *lactis, L. lactis* subsp. *diacetylactis, L. lactis* subsp. *cremoris, S. thermophilus, L. delbruecki* subsp. *bulgaricus*	Aneja et al. (2002)
40	Somar	Yak or cow milk	Buttermilk	*L. paracasei, L. lactis*	Dewan and Tamang (2007)
41	Sour milk kerbah	Milk	Acid fermented milk	*L. lactis, Sacch. kefir, Lb. casei, L. brevis, L. plantarum*	Mayo et al. (2010)
42	Sua chua	Dried skim milk, starter, sugar	Acid fermented milk	*L. bulgaricus, S. thermophilus*	Alexandraki et al. (2013)
43	Shyow	Yak milk	Acidic, thick-gel viscous, curd-like, savory	LAB, yeasts	Tamang (2010a)
44	Tarag	Cow, yak, goat milk	Acidic, sour, drink	*L. delbrueckii* subsp. *bulgaricus, L. helveticus, S. thermophilus, S. cerevisiae, Issatchenkia orientalis, Kazachstania unispora*	Watanabe and Wakasugi (2008)
45	Viili	Cow milk	Thick and sticky, sweet taste, breakfast	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis biovar, Diacetylactis, L. mesenteroides* subsp. *cremoris, G. candidum, K. marxianus, P. fermentans*	Kahala et al. (2008)
46	Wara	Milk	Sweet taste, beverage	*L. lactis, Lactobacillus* sp.	Olasupo et al. (2010)
47	Yoghurt	Animal milk	Acidic, thick-gel viscous, curd-like product, savory	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus, Lb. casei, L. rhamnosus, Lactobacillus gasseri, Lactobacillus johnsonii, Bifidobacterium* spp.	Tamime and Robinson (2007)

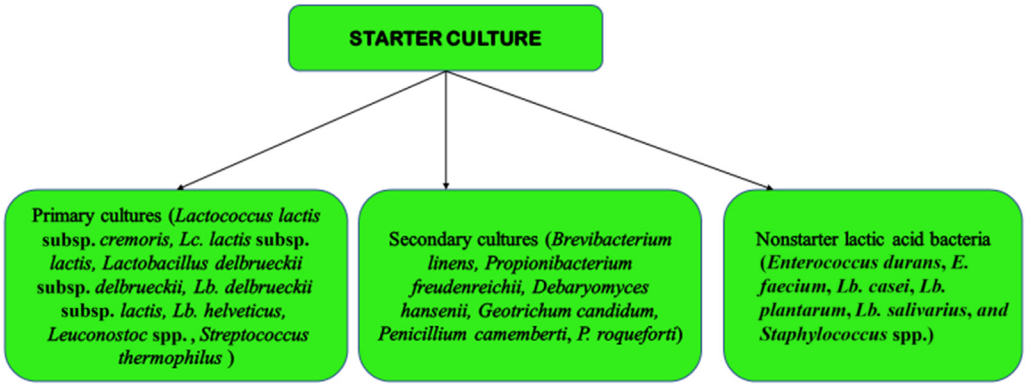

FIGURE 2.4 Type of cultures.

food fermentation process (Stevens & Nabors, 2009) and can be used to produce several compounds (enzymes, flavors, fragrances, etc.) (Longo & Sanromán, 2006). With an estimated 5000 varieties of fermented foods and beverages, worldwide, only a small fraction of these artisanal products have been subjected to scientific studies so far (Tamang, 2010b). In the beginning, starter cultures were isolates from earlier fermentations that were maintained and propagated at the site of production (Hansen, 2004; Mogensen et al., 2002). Functional microorganisms transform the chemical constituents of raw materials of plant/animal sources during fermentation thereby enhancing the nutritional value of the products, enriching them with improved flavor and texture, prolonging their shelf live, and fortifying them with health-promoting bio-active compounds (Farhad et al., 2010; Oguntoyinbo et al., 2007; Tamang, 1998). Similarly, fermentation can basically be performed either naturally or spontaneously by back-slopping or by the addition of starter cultures. By spontaneous fermentation, the raw material and its initial treatment will encourage the growth of an indigenous microbiota. Molds only grow aerobically, limiting their occurrence in certain types of fermented products. LAB produces lactic acid and other antimicrobial substances that inhibit the growth of harmful bacteria along with reducing the sugar content, thereby prolonging the shelf life of the product. Yeasts mostly produce aroma components and alcohols. When molds are involved in fermentation, they generally contribute by producing both intra- and extracellular proteolytic and lipolytic enzymes that highly influence the flavor and texture of the product (Tamang & Fleet, 2009). Fermented foods are the hubs of consortia of microbiota and mycobiota (functional, nonfunctional, and pathogenic contaminants), which may be present as natural indigenous microbiota in uncooked plant or animal substrates, utensils, containers, earthen pots, or environments (Hesseltine, 1983; Tamang, 1998), or as a result of the intentional addition of the microorganisms as starter cultures in an industrial food fermentation process (Stevens & Nabors, 2009). Next, are different types (with genera) of microorganisms associated global fermented milk (Fig. 2.5; Alexandraki et al., 2013; Bernardeau et al., 2006; Bourdichon et al., 2012; Tamang, 2010a, 2010b, 2010c; Tamang & Fleet, 2009).

2.6.1 Bacteria

Bacteria are the most dominant microorganisms in both naturally fermented foods or foods fermented by the use of starter cultures. Among the bacteria, LAB are commonly associated with acidic fermented foods, while non-LAB bacteria such as *Bacillus, Micrococcaceae, Bifidobacterium, Brachybacterium, Brevibacterium*, and *Propionibacterium* etc., are also involved in food fermentation, frequently as minor or secondary groups. Like *Acetobacter, Arthrobacter, Bacillus, Bifidobacterium, Brachybacterium, Brevibacterium, Carnobacterium, Corynebacterium, Enterobacter, Enterococcus, Gluconacetobacter, Hafnia, Halomonas, Klebsiella, Kocuria, Lactobacillus, Lactococcus, Leuconostoc, Macrococcus, Microbacterium, Micrococcus, Oenococcus, Pediococcus, Propionibacterium, Staphylococcus, Streptococcus, Streptomyces, Tetragenococcus, Weisella*, and *Zymomonas*. Bacteria is further divided into LAB and non-LAB.

2.6.1.1 Lactic acid bacteria

LAB is classified as gram-positive bacteria which include low Guanine + Cytosine (G + C) content as well as being acid tolerant, non-motile, non-spore forming and are rod- or cocci-shaped. The main function of LAB is to produce lactic acid, that is, the acidification of the food. Thus, the main application of LAB is as starter cultures. Besides, LAB contribute to the flavor, texture, and nutritional value of the fermented foods through production of aroma components,

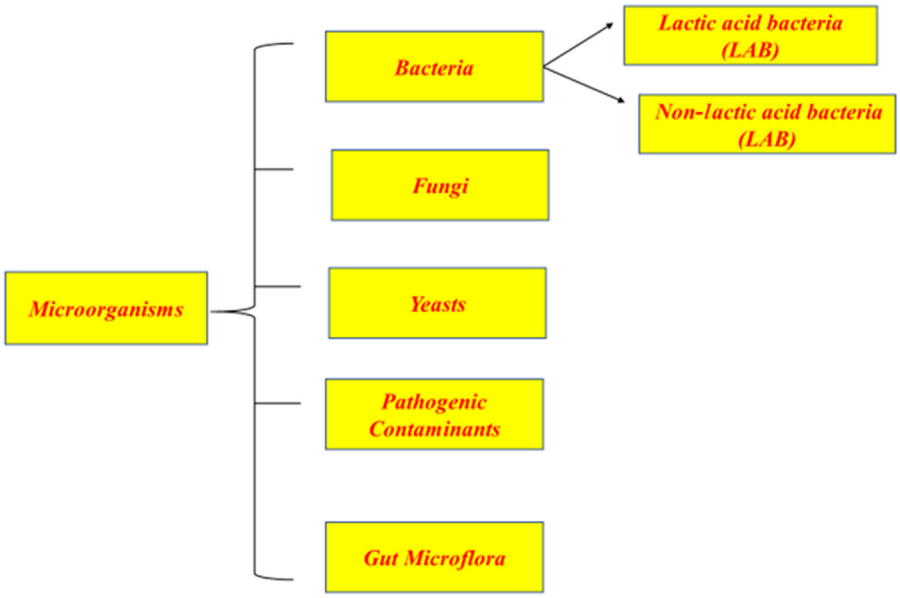

FIGURE 2.5 Microorganisms associated with milk fermentation.

and used as adjunct cultures (Ashwini et al., 2010; Batish et al., 1988); production, or degradation of exopolysaccharides, lipids, and proteins, production of nutritional components such as vitamins, and used as functional cultures, and promoting therapeutic effects and used as probiotics (Salque et al., 2012; Bahadur et al., 2020; Batish et al., 1988). In addition, they contribute to the inhibition of spoilage and pathogenic microorganisms and thus, used as bioprotective cultures (Balasubramanyam & Varadaraj, 1998). The LAB comprise a large bacterial group consisting of about 380 species in 40 genera of 6 families, belonging phylogenetically to the order Lactobacillales within the phylum Firmicutes (Stiles & Holzapfel, 1997). Common genera of the LAB isolated from various fermented foods of the world are *Alkalibacterium, Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus*, and *Weissella* (Carr et al., 2002; Salminen et al., 2004; MetaMicrobe.com/Lactic Acid Bacteria, 2013). The three main pathways which are involved in the development of flavor in fermented food products are glycolysis (fermentation of sugars), lipolysis (degradation of fat) and proteolysis (degradation of proteins) (Berger et al., 2007; Bhandari et al., 1985; Kalla et al., 2017). Lactate is the main product generated from the metabolism of lactose and a fraction of the intermediate pyruvate can alternatively be converted to diacetyl, acetoin, acetaldehyde or acetic acid (some of which can be important for typical yogurt flavors). Some LAB like *Lactobacillus* are highly utilized for health promoting bacteria activity, anti-hypertensive, calcium-binding activity, and anti-cancer.

Antimicrobial compounds

Lactic acid bacteria produces some antimicrobial metabolites during the fermentation process (Sharma et al., 2012). Many inhibitory compounds including bacteriocins and hydrogen peroxide which produced in fermentation process and work against other bacteria. LAB also help in preventing diarrheal diseases and changing the composition of microorganisms in intestines (Saxelin et al., 2005). Bacteriocins which are protein antimicrobial agents are also produces by LAB.

Use as starter cultures

Lactic acid bacteria is also used as starter strains in the manufacturing of different fermented milk products, mainly *L. lactis, L. helveticus, Streptococcus thermophiles, Lactobacillus delbruicki* subsp. Commonly used as milk starter cultures are *Bulgaricus*. *L. bulgaricus and S. thermophilus* are used in making of yogurt, and *Lactococcus casei* is found in cheeses. The company of fermented milk products needs to select a balanced amount of quality LAB used for starter cultures to make their desirable flavor and texture (Derek et al., 2009).

Used as a preservative

Generally, milk can be stored just for a few hours, while the LAB fermented milk products can be stored for the whole year (Giraffa et al., 2010). There are some varieties of cheese that can be stored for 5 years. This is the cheapest technique to preserve milk with LAB. The fermented food is more popular then unfermented according to consumers

because of its organoleptic properties. It reduces pH below 4°C because of acid production and it stops pathogenic microorganism growth. These microorganisms can produce disease and spoil milk (Ananou et al., 2007).

2.6.1.2 Non-lactic acid bacteria

Bacillus is reported from the alkaline-fermented foods of Asia and Africa (Parkouda et al., 2009). Species of *Bacillus* present in fermented foods mostly soybean-based foods are *B. amyloliquefaciens*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis, B. subtilis variety natto and B. thuringiensis* (Kiers et al., 2000; Kubo et al., 2011), while strains of *B. cereus* have been isolated from the fermentation of *Prosopis africana* seeds for the production of okpehe in Nigeria (Meerak et al., 2007; Nishito et al., 2010; Oguntoyinbo et al., 2007; Urushibata et al., 2002). Species of *Bifidobacterium, Brachybacterium, Brevibacterium*, and *Propionibacterium* have been isolated from cheese and other fermented milks (Bourdichon et al., 2012; Coton et al., 2010; Martín et al., 2006; Wu et al., 2000).

2.6.1.3 Fungi

The major roles of fungi in fermented foods and alcoholic beverages are the production of intra- and extracellular proteolytic and lipolytic enzymes that highly influence the flavor and texture of the product, and also the degradation of anti-nutritive factors improving bioavailability of minerals (Aidoo & Nout, 2010; Josephsen & Jespersen, 2004). Species of *Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora, Penicillium, Rhizopus*, and *Ustilago* are reported from many fermented foods, asian nonfood amylolytic starters and alcoholic beverages (Hesseltine, 1991; Nout & Aidoo, 2002). Examples include *Actinomucor, Aspergillus, Fusarium, Lecanicillium, Mucor, Neurospora, Penicillium, Rhizopus, Scopulariopsis*, and *Sperendonema*.

2.6.1.4 Yeasts

The role of yeasts in food fermentation is to ferment sugar, produce secondary metabolites, inhibit growth of mycotoxin-producing molds and display several enzymatic activities such as lipolytic, proteolytic, pectinolytic, glycosidasic and urease activities (Aidoo et al., 2006; Romano et al., 2006). Genera of yeasts reported from fermented foods, alcoholic beverages and nonfood mixed amylolytic starters are *Brettanomyces, Candida, Cryptococcus, Debaryomyces, Dekkera, Galactomyces, Geotrichum, Hansenula, Hanseniaspora, Hyphopichia, Issatchenkia, Kazachstania, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Rhodosporidium, Saccharomyces, Saccharomycodes, Saccharomycopsis, Schizosaccharomyces, Sporobolomyces, Torulaspora, Torulopsis, Trichosporon, Yarrowia*, and *Zygosaccharomyces* (Kurtzman et al., 2011; Lv et al., 2013; Tamang & Fleet, 2009; Watanabe & Wakasugi, 2008).

2.6.1.5 Pathogenic contaminants

About 80% of fermented foods are produced by natural fermentation and may contain functional, nonfunctional, and pathogenic microorganisms during initial fermentation. Pathogenic bacteria commonly reported for fermented foods are *Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica, Bacillus cereus*, and *Clostridium botulinum* (Lindqvist & Lindblad, 2009; Rossi et al., 2011).

2.7 Ecology of fermented microorganism

Fermentation is a biological food processing technique and growth of nontoxigenic beneficial microorganisms is desirable. The microorganisms used may already existent in food (i.e., cabbage for sauerkraut) or added as starter culture (i.e. inoculating milk for yogurt) (De Vuyst et al., 2014). Without the use of starter cultures, there is more variability in the bacteria present and potential for less consistency with the end product (Johanningsmeier et al., 2007; Peñas et al., 2010). Fermentation occurs through an anaerobic conversion of carbohydrates by microorganisms to the metabolic end products. There are two kingdoms of fermenting microorganisms: fungi, which are multicellular and include yeast and molds, and unicellular eubacteria. Most of the fermenting bacteria are functionally classified as LAB because they are carbohydrate-fermenting bacteria that produce lactic acid as a major metabolic end product (Holzapfel & Wood, 2014). LAB are categorized as either homolactic or heterolactic contingent on the metabolic pathway and end products, primarily determined by family. Homolactic bacteria follow the glycolysis or Embden-Meyerhof-Parnas (EMP) fermentation pathway; whereas, heterolactic bacteria follow the phosphoketolase pathway. Homofermenters primarily include families Enterococcaceae, Lactobacillaceae, and Streptococcaceae, and convert one mole of glucose to two moles of pyruvate, which are reduced to lactic acid in the absence of oxygen. Heterofermenters primarily include families

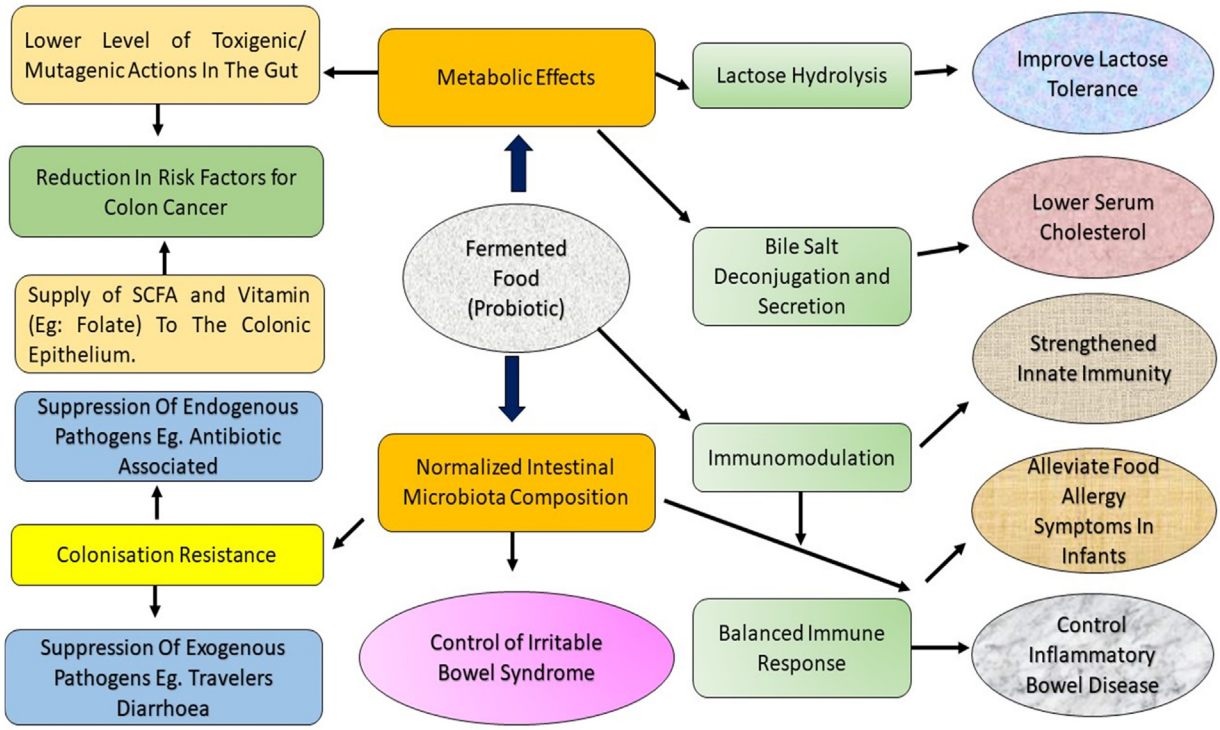

FIGURE 2.6 Ecology of fermented microorganism.

Leuconostocaceae and species within the *Lactobacillus* genera. Following this pathway utilizes one mole of glucose to produce one mole each of lactic acid, carbon dioxide, and ethanol (Endo & Dicks, 2014). LAB are social microorganisms, and fermenting the substrate is part of the practical relationship to foster a suitable environment for bacterial colony growth and development. There are three main stages of bacterial sequencing, as one bacteria starts to slow down due to increased acidity, conditions become favorable for the next in sequential colonization, yet enzymes still function (Battcock & Azam-Ali, 1998; Fig. 2.6).

2.8 Types of dairy products

Another broad classification of fermented milks given by Batish et al. (2001) is depicted in Fig. 2.7. There are various types of fermented milk and its products which have been developed in all over the world with its own characteristic's history. Quality of these products depends very much on the type of milk, on the temperature, fermentation condition and on the technology treatments. Commonly used dairy products are curd, cheese, yogurt, kefir and kumin (Wouters et al., 2002).

2.8.1 Dairy products

Dietetic fermented dairy drinks are a traditional mesophilic fermented milk beverage of India. It is essentially prepared by blending dahi with salt, spices, and certain cereals. Since, there is scarcity of literature on fermented dairy drinks, thus relevant research efforts in comparable western products like yogurts, stirred yogurts, yogurt beverages, yogurt drinks, and fermented milks in general, have also been reviewed. Dairy products are a very good source of calcium, vitamin D, protein, and other essential nitrites. These dairy products are also rich in phosphorus, potassium, magnesium, and various vitamins like vitamin A and vitamin B12. Lots of dairy products are prepared by using different microbial strains. Microorganisms help to ferment the carbohydrate present in milk, which is mainly lactose, to lactic acid and some other products. In the milk, the acid precipitates the proteins; therefore, all the fermented products are thicker in consistency than milk (De los et al., 2015). Nowadays lots of variety of fermented dairy products are available for the consumers. Although a small proportion of these products are homemade, most of them are produced industrially (Kumar & Chordia, 2017).

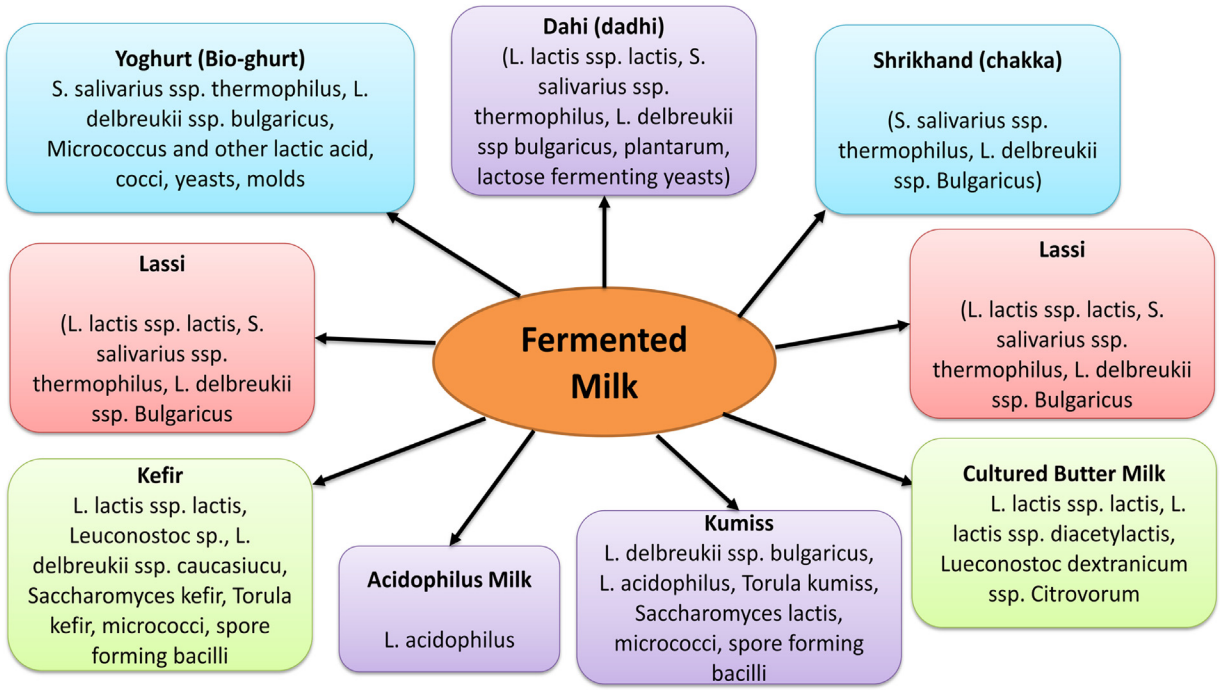

FIGURE 2.7 Classification of fermented milks.

2.8.2 Curd

It is made by curdling or coagulating the milk. This process can be done by mixing edible acidic substances in to the milk, like vinegar or juice. In this process, milk will curdle and separate into two parts. One part is liquid that is whey and the solid part is curd. It is the milk proteins or casein. Sometimes without adding any acidic substance, old milk might get soured. This is happening because raw milk contains *Lactobacillus*. This bacterium converts lactose of the milk into lactic acid which imparts the sour taste to curd (Ledenbach & Marshall, 2009; Quigley et al., 2011).

2.8.3 Yogurt

One of the most commonly used dairy products is yogurt. It is prepared by heating the milk to nearly 80°C (176 Ferenhite) in order to kill any additional bacteria that may be present and to denature milk proteins. Then the milk is allowed to cool to 45°C; after that, it is inoculated with bacteria and allowed to ferment at room temperature. The bacteria used for this process are *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. Sometimes when bacteria are not available then a spoonful of yogurt can also be added because it contains bacteria. Probiotic bacteria like *S. thermophilus, L. acidophilus* and *bifidobacteria* can also be used for the production of yogurt and it is commonly referred as bio-yogurt (Lourens-Hattingh & Viljoen, 2001). Researchers also said that consumption of probiotic microorganisms helps to maintain a suitable microbial profile and results in several remedial benefits (Kumar & Chordia, 2017). There are many factors that affect the quality of yogurt in industries: choice of milk, milk additives, deaeration, heat treatment, choice of culture, and plant design. The milk which is used in preparation of yogurt must be the highest bacteriological quality. Make sure that milk does not contain any type of antibiotics, bacteriophages, or sterilizing agents. Milk should be homogenized and the air content of milk should be as low as possible; milk should be heated before the inoculation (Soukoulis et al., 2007; Fig. 2.8).

2.8.4 Cheese

It is a fermented milk product. The process of making cheese has three main steps; in first step, milk is molded into solid curd and liquid whey by coagulation of protein (casein) present in milk. In the process of coagulation of protein (casein) acidification and proteolysis is done. When LAB ferment the disaccharide lactose to produce lactic acid, this process is called acidification. Nowadays, the acidification process is done by the bacterial culture strain of *L. lactis, S.*

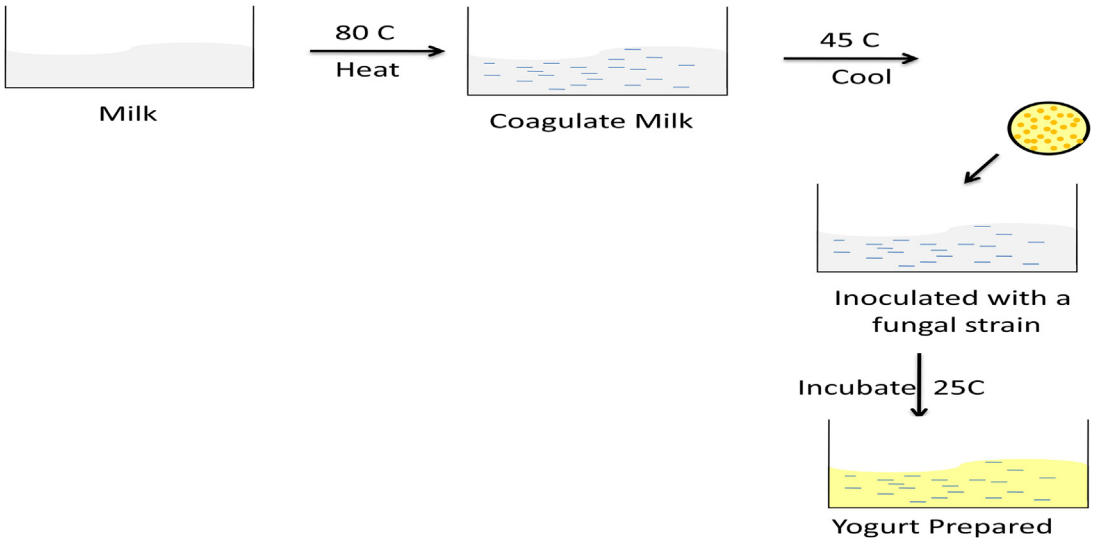

FIGURE 2.8 The process of preparing yogurt.

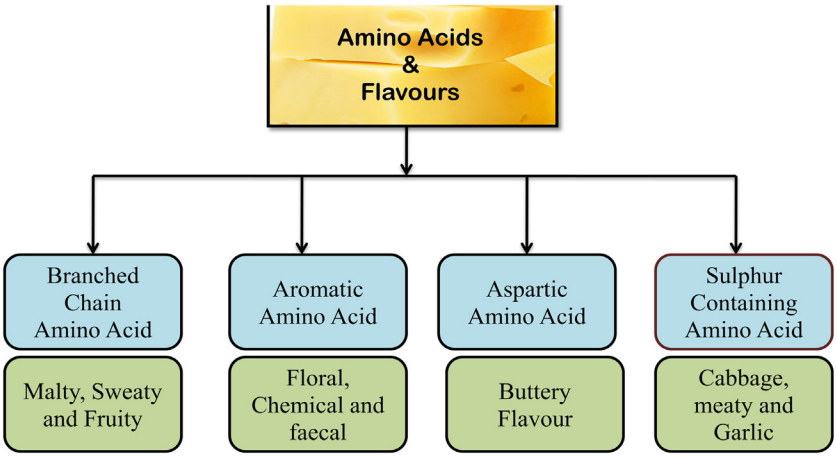

FIGURE 2.9 Amino acids are responsible for flavor in cheese.

thermophilus and *Lactobacillus* sp. In the second step, curd that contains the casein and milk fat is separated from the whey. The curd can be heated, salted, pressed, and is molded into various shapes and sizes; it all depends on the type of cheese. After that, the interior part of cheese metabolizes by growing LAB and cultures, while the surface of cheese is colonized by bacteria and fungi that create a bio-film of multispecies called rind of cheese (Button & Dutton, 2012). Flavor, smell, and texture of cheese are because of some different microbes. Amino acid plays an important role for the flavor of any cheese. In cheese lactic acid bacteria and other microorganisms degrades amino acid to aromatic compounds. There are different amino acids responsible for different flavors are given below (Ardo, 2006; Yvon & Rijnen, 2001; Fig. 2.9).

2.8.5 Butter

Butter is made by the separated cream of milk which is soured. This souring of the cream is brought about by organisms naturally present in the milk, or first the milk may be pasteurized and then microorganisms are added it. Microorganisms that help to making butter are *Streptococcus lactis* and *Streptococcus cremoris;* these ferment the lactose and sour the cream, other streptococcal-like organisms, *Leuconostoc citrovorum* and *L. dextranicum* are also present and add their particular flavors to the butter. Temperature is also an important factor in making butter; at which

temperature the cream is soured is very important. Temperatures around 20°C are adequate to prevent growth of thermophilic spoilage organisms, which have survived pasteurization, and yet are suitably high enough to allow growth of the desirable *streptococci*. In this process of making butter, the soured cream is churned. With the help of that churned milk, the fat globules aggregate to form butter, leaving the buttermilk which can be drained off (Kalla et al., 2017).

2.8.6 Kefir

It is a fermented dairy product drink which has its origin in Eastern Europe. This is light alcoholic beverage prepared by inoculation of raw milk with irregularly shaped, gelatinous white/yellow grain called kefir grains. Diversity of microbes are present in yeasts, LAB, acetic acid bacteria, and mycelial fungi.

2.9 Role of advance biotechnology in fermentation technology

There are some applications of biotechnology in dairy industry. Some of possible applications are Recombinant bovine, recombinant vaccines, DNA fingerprinting, embryo transmit, technology, animal cloning and gene forming and transgenic, food grade biopreservatives, dairy enzymes/proteins, probiotics, functional foods and nutraceuticals, dairy waste organization, and pollution control (Ramchandran et al., 2009) in biotechnology enzyme are to formed high quality food material. There are many applications employed every day at home. Some of these applications are enzymes used to make various improvements in the quality of diverse foods. Enzymes are necessary for the manufacture of cheese yogurt and other milk products (Loly, 2011; Papademas et al., 2013; Schaafsma et al., 2008). Novel starter cultures are continually in demand for the development of new commercial goods, along with greater characterization of those currently in use, to ensure safe and functional products (Hill et al., 2017). There are many positive and negative factors that impact the selection of starter cultures in dairy fermentations, such as a history of safe use, acidification rate during fermentation, exopolysaccharide production (Ryan et al., 2015), proteolytic activity particularly during cheese production, and the generation of bioactive metabolites and peptides (Dobson et al., 2012; Pessione & Cirrincione, 2016). Fermentation starter cultures can produce a number of desirable and undesirable bioactive metabolites. For example, biogenic amines (histamine, tyramine) are s undesirable products in most fermentations due to their toxicity. Bioactive peptides produced through enzymatic release are desirable by-products due to positive biological activity. Bacteriocins are desirable as a known probiotic trait, but potentially undesirable in a starter culture due to possible impact on other fermenting cultures (Hill et al., 2017; Fig. 2.10). Recently, a wide range of functional foods were developed and varieties of them are being produced all over the world including probiotic, probiotic, synbiotic foods as well as foods enriched with fat-reduced, salt-reduced or sugar-reduced foods, antioxidants, and phytosterols. All these foods are beneficial, but probiotic functional food has extended positive effects on our health (Kampman et al., 2000).

2.10 Factors affecting quality of dairy drink

The quality of fermented milk depends on the composition and processing parameters as well as the subsequent handling and storage of the product.

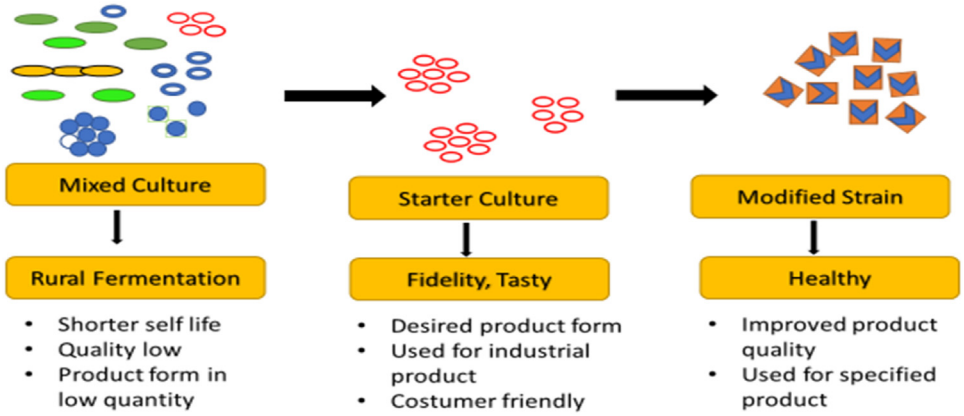

FIGURE 2.10 Role of biotechnology in fermentation technology.

2.10.1 Quality of raw milk

Riber (1995) found that milk for fermented milk manufacture should be of excellent bacteriological quality, free from mastitic organisms with low SPC, somatic cell count, and psychrotrophic count. It should be free from antibiotics, lipolytic rancidity, residual antibiotics, germicides and bacteriophage contamination, and sanitizer residue or any other inhibitory substances or late lactation milk (Tiwari, 1997). Liquid milk may also contain cellular material like epithelial cells and leukocytes that originate from the udder of bovine animals, which is in some instances due to carelessness during milk production (Tamime & Robinson, 1999). The milk is prone to further contamination with straw, leaves, hair, soil, seeds, etc. (Patnaik, 2003; Fig. 2.11).

2.10.2 Type of raw milk

The dahi made from buffalo milk had higher titratable acidity (TA) as compared to cow milk dahi and skim milk dahi which showed more whey separation than whole milk dahi (Bhandari et al., 1985; Sharma et al., 1974). Mixed cow milk and buffalo milk gave lassi better flavor and texture (Patidar & Prajapati, 1998). Dahi forms a weak gel when prepared from cow milk, whereas buffalo milk yields a firm gel owing to its high total solids (TS).

2.10.3 Homogenization

Homogenization is carried out to obtain a uniform distribution of fat globules and prevent separation of fat (Samuelsson & Christiansen, 1978). It may lead to improvement in viscosity of resultant fermented milk, tend to reduce syneresis and increase water holding capacity. The advantageous physical − chemical changes caused by homogenization of the milk base during the manufacture of fermented milk, include: (1) a reduction in the diameter of fat to <2 mm, these small globules being less inclined to coalesce into larger units and rise to the surface; (2) Milk becomes whiter in color due to enhanced light reflectance; (3) an increase in viscosity of the product due to interaction and/or adsorption of the fat globules onto the casein micelles; and (4) a decrease in syneresis of the gel due to increase in its hydrophilicity and waterholding capacity as a result of the interaction(s) of the proteins (Tamime, 2002).

2.10.4 Starter culture

The role of microbes in producing fermented dairy products has evolved from a chance discovery to a highly elaborated process involving the production of specialized "starter" of bacteria that function consistently in large cultures (Aneja et al., 2001). The primary function of almost all starter cultures is to develop acid in the product. The secondary effects of acid production include coagulation, expulsion of moisture, texture formation and initiation of flavor production (Batish et al., 2001).

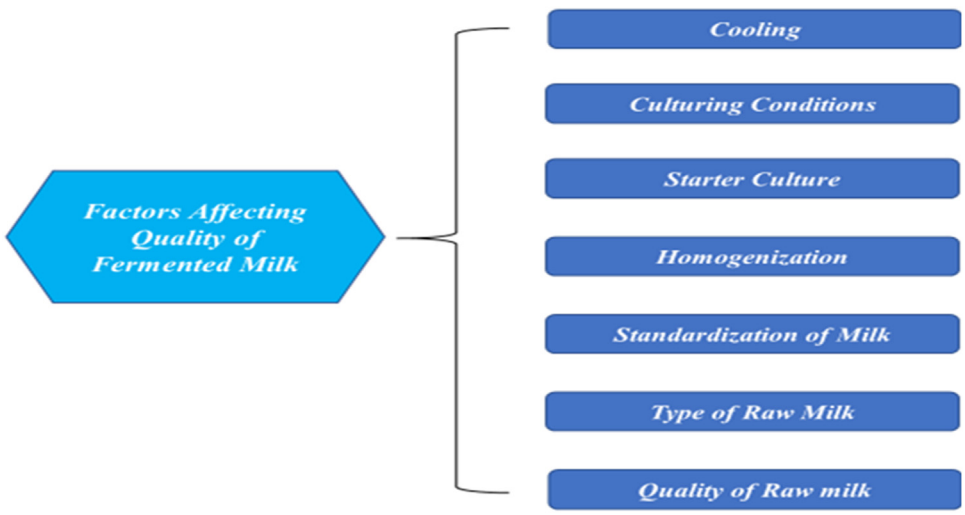

FIGURE 2.11 Factors affecting quality of dairy drink.

2.10.5 Culturing conditions

LAB during milk fermentation can be influenced by a number of factors (Batish et al., 2001) like culturing time (Towler, 1984), temperature (Bhandari et al., 1985) pH, strain compatibility, growth medium, inhibitors, presence of bacteriophages, incubation period, heat treatment of milk, degree of aeration, effect of carbon dioxide, and storage conditions (Aneja et al., 2001). some examples include: (1) yogurt at 40°C−45°C for up to 3 hours, at 30°C for 18 hours or 5 hours when using direct-to-vat inoculation (DVI) starter culture; (2) probiotic products at 37°C for a few hours or up to 5−7 days depending on the organism(s) used; (3) buttermilk at 20°C−30°C for up to 10−20 hours; (4) kefir at 22°C for 16−24 hours depending on the type of kefir grains used (Tamime & Marshall, 1997; Wszolek et al., 2001) and (5) dahi at 20°C−28°C for 14−16 hours (Harris, 1990; Imeson, 1997; Kumar & Solanky, 1997; Phillips & Williams, 2000; Tamime & Robinson, 1999). Stabilizers also play a important role in fermentation. Stabilizers can be categorized according to the manufacturing process (Tamime & Robinson, 1999). Hydrocolloids can either be of natural, modified, or synthetic origin. Selection of the stabilizer or stabilizer combination to be used in a food system greatly depends on several variables.

2.10.6 Cooling

Fermented dairy drink production is a biological process and cooling is one of the popular methods to control metabolic activity of the starter culture and its enzymes (Afonso & Maia, 1999; Tamime & Robinson, 1999). This increase in texture depends on the speed of the yogurt cooling. Slow cooling permits an enhanced texture, evaluated by flowing properties and firmness (Aneja et al., 2001).

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Chapter 3

Recent trends in fungal dairy fermented foods

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3.1 Introduction

Fermented foods have been occupying a significant position on the human's meal table for thousands of years. Their journey started when man discovered food fermentation just as a means of preservation, completely unaware of the desirable health attributes and organoleptic properties that fermentation offered. Gradually, the unique flavors as well as health-promoting effects of fermented foods were uncovered, leading to the intentional production of fermented foods as dependable sources of various important nutrients like vitamins, minerals, etc., even before the emergence of nutrition science (Steinkraus, 1994). Depending on the substrates and raw materials from which they are prepared, these fermented foods offer peculiar flavors, appearances, textures, and other functional characteristics. Additionally, an improved microbial flora and safety are also offered by few fermented foods to such an extent that some of them can even be stored at ambient temperatures. Hence, fermentation can be employed to maintain and improve the shelf-life, texture, and flavor, as well as the overall functionalities of foods (Hutkins, 2018). Milk is known as a complete food, but wherever milk is utilized as food, its fermented variants undoubtedly were prepared, accepted, and consumed. Therefore, globally, these fermented dairy food products, today contribute around 20% to the total economic value of fermented food items. Although the time and place of first practice in dairy husbandry is unknown, the milk's nutritious constitution has been providing substantial benefits since human evolution. However, the same components in milk that make it wholesome for human consumption also invite microbial contaminants in it that makes milk an extremely perishable food item. Hence, fermentation developed as the paramount way to increase the shelf-life of milk and to improve its nutritive value. In 1907, Metchnikoff advocated that the gut microbiota had detrimental repercussions on human health and named it autointoxication. He further proposed that the consumption of fermented milk products improved this situation. Thereafter, for the treatment of constipation, he employed intestinal strains of *Lactobacillus acidophilus* based on the assumption that gut colonization is obligatory for paramount favorable health effects (Fuller, 1991; Rettger & Chaplin, 1921). The fermented milks make up a very significant part of the human diet due to their beneficial antimicrobial, hypotensive, and hypo-cholesterolemic consequences (Ohsawa et al., 2015; Shiby & Mishra, 2013) in addition to their ability to modulate gut microbiota and the immune system. The health-promoting results of fermented milks has lead to an extensive study of their functional and microbial properties (Rhee et al., 2011).

3.2 Status of milk production in India and assorted fermented dairy foods

Globally, India stands first in milk production at the world's milk output and trade market. The milk production in India has enormously increased from 17 metric ton (MT) in 1951 to 176.27 MT in 2017−18. Moreover, an increase of 5.6% in milk production has been achieved in 2018−19 with total milk production of 186.143 MT. Thus, by disseminating awareness among dairy farmers as well as to other dairy market players backed with government support, a vast market of fermented milk products' can be built in India. With more than 400 generic names, several variants of traditional as well as commercial fermented dairy products are now available worldwide. Apart from these, there also exist at least 1000 cheese varieties and several variants of cheese-like products too. Classification of fermented dairy foods

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can be done based on various factors, such as the coagulation method (enzymatic, acidic, and acid plus heat); method of whey separartion and the amount separated (from minimum to complete); and by the nature of fermenting microbes (pure lactic acid bacteria or consortium of lactic acid bacteria and fungi or fungi alone).

3.2.1 Cultured milk products

3.2.1.1 Naturally fermented milks

The naturally fermented milk products are prepared by allowing the fermentation of indigenous microflora present in milk, without using any starter culture. These are the simplest and possibly the ancient type of fermented dairy products produced around the globe. The spontaneous fermentation in such milks depends upon the microbiota present in their raw material and in the environment, which includes workers, animals, as well as equipment (Franz et al., 2014). These indigenously fermented dairy food items are still popular in various countries and regions. For example, in the Middle East and Northern African, leben, laban, kad, rayeb, zeer and zabady, are popular naturally fermented dairy products. Moreover ergo, roub, amasi, and filmjölk along with långfil are important spontaneously fermented dairy foods found in Ethiopia, Sudan, Zimbabwe, and Sweden respectively (Tamang et al., 2016).

3.2.2 Starter culture-dependent fermented milks

These are the fermented milk products prepared by inoculating a particular starter culture in them. This category of fermented milks has become prevalent, and among these yogurt and associated products are the most commonly known.

3.2.2.1 Yogurt

It is a fermented and coagulated dairy product containing a higher concentration of protein, vitamins (B_2 and B_{12}), calcium, potassium, zinc, and magnesium as compared to the milk (Wang et al., 2013).

3.2.2.2 Dahi

It is a yogurt-like fermented dairy product found particularly in India, Sri Lanka, Bangladesh, Nepal, and Pakistan. Dahi is found to be mentioned from 6000 to 4000 BCE in the Rigveda and Upanishad. A similar product, named as yogurt, was being prepared by the Turkish people residing in Asia (Rasic & Kurmann, 1978). Other notable examples of dahi-like products from India include chhu, chhurpi, churkam, mar, and gee (Dewan & Tamang, 2006; Shangpliang et al., 2018), while in Egypt Zabady is a commonly found dahi-like fermented milk item (El-Baradei et al., 2008).

3.2.2.3 Acidophilus milk

It is another fermented dairy product, which is prepared using *L. acidophilus* as a starter culture. The acidophilus milk can either be fermented or non-fermented. In the former case, milk is heat processed to 95°C−120°C followed by its inoculation (2%−5% inoculant) at 37°C. After incubation, the product is stored at a low temperature (5°C). In unfermented acidophilus milk, the culture is not allowed to ferment milk at ambient conditions; rather, the commercial pure culture of *L. acidophilus* is inoculated to cold milk at 5°C−7°C, followed by its incubation again at low temperature (Kurmann et al., 1992; Shiby & Mishra, 2013).

3.2.2.4 Bifidus milk

It was being produced as the first infant product in 1948 by Mayer in Germany. During its production, the mixed bacterial culture of *Bifidobacterium longum* and *Bifidobacterium bifidum* is inoculated (@10%) to milk at ambient temperature. The commercial, ready to use bifidus milk comprises 10−100 million *Bifidobacterium* per gram and the product has a peculiar spicy and acidic character in its aroma (Kurmann et al., 1992).

3.2.2.5 Acidophilus-bifidus milk (AB culture)

It is another dairy product fermented using 1:1 culture of *L. acidophilus* and *Bifidobacteium* sp. (Kurmann et al., 1992; Ozden, 2008).

Some other traditional but globally popular fermented milk products include Bulgarian buttermilk, koumis, kefir, and villi (Ramesh et al., 2006). Kefir and koumis are spontaneously fermented milk products from Balkan-Caucasian and Caucasian origin, respectively.

3.2.2.6 Bulgarian buttermilk

It is a fermented milk product pointedly having more tartness than yogurt.

3.2.2.7 Kefir

It is a viscid, tart, lightly alcoholic, and mildly effervescent milk beverage fermented with kefir grains. The kefir grains are composed of a polysaccharide material maintaining a substantial microbial community, and after the completion of fermentation, these grains can be used again (Prado et al., 2015).

3.2.2.8 Koumiss

It is specified by using unpasteurized mare's milk for fermentation. This milk, as a consequence of its lower protein but higher fat content, imparts into the product a nice and rich taste as well as a smooth consistency. Koumis is reported to be first used by Mongols to cure diseases like ulcers, hepatitis, and tuberculosis. Although originated with Asian nomads, koumis is still widely utilized in central and western Asian countries like Russia, Kyrgyzstan, Kazakhstan, and Mongolia.

3.2.2.9 Acidophilin

It is an acidic fermented milk product, a produce of cow's milk fermentation with a consortium of *L. acidophilus, Lactococcus lactis* ssp. *lactis* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Sometimes, the starter culture of kefir is also added along with the starter culture of acidophilin, so that the finished product contains around 97% *L. lactis* ssp. *lactis*, 2% *L. acidophilus* and 1% yeast and around 0.67%−1.08% acidity (Kurmann et al., 1992).

Similar fermented products, airag and chigee, are produced widely in northwestern China and Mongolia. Another related but very well-known fermented milk product is villi of Finland characterized by its viscous and sticky consistency. For the fermentation of starter culture-dependent fermented milks, commercial cultures are available these days. However, for conventional fermented products, the starter culture is obtained from a preceding batch and this technique of inoculation is known as back-slopping method.

3.2.2.10 Mil-mil and yakult

These are two commercially produced fermented dairy products of Japan. The starter culture of mil-mil comprises the consortium of *B. bifidum*, *Bifidobacterium breve*, and *L. acidophilus*. The final product is enriched with defined amounts of carrot juice, glucose, and fructose, which makes it rich in vitamin A (Kurmann et al., 1992). Yakult, a probiotic milk product, is prepared by employing a specific strain of *Lactobacillus*, known as *L. casei* Shirota strain (named after its discoverer).

3.2.2.11 Cheese

It is a fermented milk product, obtained by milk coagulation and whey separation. These are high-quality fermented products having a higher fat content, energy, calcium, protein, and vitamin B content (Ansorena & Astiasarán, 2015). In the process of cheese production, milk casein is broken down with a starter culture, peptidases, and proteases from a secondary microbial flora. Cheese can either be consumed fresh or stored for a long time, provided it is salted and/or dehydrated. Based on the mode of coagulation, cheeses can be categorized as:

Acid-coagulated cheese

1. It includes cream cheese, cottage cheese, and quarg which accounts for around 25% of total cheese consumption.

Acid plus heat coagulated

1. It includes Ricotta cheese.

Enzymatic coagulated

1. The enzymatic coagulation of milk is done by using rennet, original or genetically engineered chymosin, acid proteinases, and some other enzymes.
2. Cheeses can be further classified based on their particular method of manufacture, mode of ripening, and moisture content in them.

Soft to hard bacterial internally ripened cheese

1. This class includes parmesan (very hard), cheddar (hard), Emmental (eyed cheese), Edam, and Gouda cheese (Dutch types). Feta (heavily salted) and mozzarella cheeses though generally not ripened may be included in this group.

Bacterial smeared cheese (surface ripened)

1. It includes Muenster, Limburger and Tilsit.

Mold ripened cheese

1. Mold ripened cheese varieties include those ripened with surface fungi (Camembert, Brie) and others which are internally ripened with fungi (Roquefort, Gorgonzola, Stilton, Cabrales). Evidence of cheese production and consumption has been recorded around 5500 BCE in Poland (Salque et al., 2013). Nowadays, although most of these cheeses are produced widely around the globe, they are often related to specific regions or countries.

3.3 Microorganisms in dairy fermented foods with reference to fungi

3.3.1 Yeasts: A brief overview of yeasts and their role in dairy fermentation

The favorable yeasts for appealing food fermentation generally belong to the *Saccharomyces* family, especially *Saccharomyces cerevisiae*. Yeasts play a significant role in the dairy food industry as a result of their enzymes produced that result in desirable biochemical reactions such as the production of ethanol in koumiss and kefir and coagulation of milk in cheese. Yeast strains like *S. cerevisiae* and *Saccharomyces boulardii* also represent the non-bacterial, eukaryotic, commercially available probiotic products in the market. The added advantage of exploiting yeasts as probiotic products lies in their ability like all eukaryotes to carry out post-translational modifications of proteins which might allow the expression of several important therapeutic proteins (Hudson et al., 2016). Moreover, significant probiotic properties like bile and acid-tolerant (Cho et al., 2018) as well as antimicrobial activity (Gotcheva et al., 2002) are reported to be present in certain yeast strains. Not only this, probiotic yeasts have been proven as successful candidates to be used along with antibiotics and this is because they possess an ability to resist many common antibiotics (Neut et al., 2017). Hence, like bacteria, yeasts are also antibiotic-resistant, but the latter cannot spread the resistance due to the location of such genes on yeasts' chromosomes (Czerucka et al., 2007). This attribute makes *S. boulardii* a preferred probiotic candidate than *L. lactis*.

3.3.2 Saccharomyces cerevisiae boulardii

It is the first discovered probiotic yeast. French researcher Boulard discovered this gram-positive yeast belonging to the Saccharomycetaceae family, in 1923 from a tropical fruit, lychee. The yeast is elliptical or spherical in shape and its size varies from 4 to 8 μm. This ascosporic yeast grows in standard yeast media having 37°C as its optimal growth temperature and can assimilate and ferment a variety of carbohydrates. *S. boulardii*, itself is a non-pathogenic yeast, but it has antimicrobial properties against pathogenic microbes' especially enteropathogenic ones. In 1962, the commercial lyophilized culture of *S. boulardii*, was prepared and particularly in France, was used to cure diarrhea. About preclinical and experimental data, *S. boulardii*, has been shown to have antimicrobial, antitoxic, antiinflammatory, and many enzymatic activities. Therefore, presently Europe, Asia, Africa, and South America are utilizing the lyophilized culture of *S. boulardii* for their clinical purposes (Billoo et al., 2006; Ertor, 2003; Szajewska, 2012). *S. boulardii* is shown to have many probiotic effects, and is used as a model organism to study the probiotic effects of yeasts. According to Moré and Swidsinski (2015), the following are the main probiotic effects/benefits offered by *S. boulardii*.

1. Stimulation of immune response: *S. boulardii* produces β(1,3)D-glucans as part of its cell wall components. These glucans, in fact, act as biological response modifiers by binding to receptors of either innate immune cells (complement receptor-3, tog like receptors) or dendritic cells like dectin-1
2. Antiinflammatory and antisecretory effects: *S. boulardii* interferes with those signaling pathways (MAP and NF-kB) in enterocytes, which regulate the functioning of the tight junction barrier and the process of inflammation.
3. A general prebiotic effect: The yeast's cell wall components like glucans, chitin, and mannoproteins are some chosen substrates for gut microbiota.
4. A positive trophic action on enterocytes: Polyamines produced by the yeast promote maturation, cell differentiation, and proliferation of the host's enterocytes.
5. Limiting pathogen binding and nullifying bacterial toxins: Some important pathogens like *Escherichia coli*, *Salmonella typhimurium, Salmonella Typhi*, *Candida albicans* are found to be inhibited by *S. boulardii*. The yeast is

also found to stably bind to some harmful compounds produced by *Clostridium difficile* and other toxins like cholera toxin.

6. Prevention of bacterial colonization by forming a physical barrier: *S. boulardii* acts as a physical barrier against pathogens by growing and forming a shielding cover on mucus. Consequently, it prevents pathogenic bacteria to inhabit the mucosal layer.

Although only *S. boulardii* enjoys the status of a probiotic yeast with commercial acceptance and having a regulatory framework, various other promising probiotic yeast candidates have also been reported by researchers Tables 3.1 and 3.2.

3.4 Yeast fermented dairy products

3.4.1 Koumiss

Its probiotic group is comprised of lactic acid bacteria (LAB) (*L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus*), lactose-fermenting yeast (*Saccharomyces* spp. *Kluyveromyces marxianus* var. *marxianus* and *Candida koumiss*), non-lactose-fermenting yeast (*Saccharomyces cartilaginous*), and non-carbohydrate-fermenting yeast (*Mycoderma* sp.). While the LAB in koumiss converts lactose to lactic acid, the yeast present in it transforms sugar into carbon dioxide and ethyl alcohol. Koumiss undergoes mainly two types of fermentation, namely lactic acid fermentation (caused by LAB) and alcohol fermentation (due to the yeast) (Chen et al., 2010); these transformations produce a peculiar sour, alcoholic flavor (Choi et al., 2016; Lv & Wang, 2009). This fermented beverage generally contains around 2% alcohol, 0.5%−1.5% lactic acid, 2%−4% lactose, and 2% fat (Mu et al., 2012; Sun et al., 2009). Moreover, it is rich in vitamins (C, A, E, D, B_1, B_2, B_{12}), trace elements, and antibiotics (Abdel-Salam et al., 2010; Dönmez et al., 2014). Koumiss is considered a complete food with several health benefits like improving gastric and intestinal health, healing the immune, nervous, and cardiovascular system of the body (Zhang & Zhang, 2012).

TABLE 3.1 List of other yeast containing fermented milk products.

Yeast containing fermented milk product	Location
Yogurt	Middle East
Dahi and mishti dahi	India
Acidophilus yeast milk, Busa, Kuban, Kurunga, Prohlada, and Salomat	Russia
Rob	Egypt
Omaere	Africa
Skyr	Iceland
Samokisselis	Yugoslavia
Airan and Arsa	Asia
Aker	Tibet
Airag, Khoormog, Tschigan, and Umdaa	Mongolia
Matzoon	Armenia
Brano milk	Bulgaria
Felisowka	Poland
Galazyme	France
Cellarmilk	Norway
Hooslanka, Urda, and Zhentitsa	Carpathian Mountains

TABLE 3.2 List of probiotic yeast strains.

Yeast strain	References
Saccharomyces cerevisiae, S. cerevisiae var. *boulardii*	Diosma et al. (2014), Gil-Rodríguez et al. (2015), Pienaar et al. (2015), Song et al. (2014)
Saccharomyces unisporus	Kourelis et al. (2010)
Cryptococcus sp.	Aloglu et al. (2015)
Candida famata	Al-Seraih et al. (2015)
Candida tropicalis	Ogunremi et al. (2015)
Debaromyces hansenii	Ochangco et al. (2016)
Issatchenkia orientalis	Ogunremi et al. (2015)
Kluyveromyces lactis	Binetti et al. (2013)
Kluyveromyces marxianus	Binetti et al. (2013), Diosma et al. (2014), Smith et al. (2015)
Metschnikowia gruessii	Smith et al. (2015)
Pichia jadinii	Buerth et al. (2016)
Pichia kluyveri, Pichia kudriavzevii	Ogunremi et al. (2015)
Pichia pastoris	França et al. (2015)
Pichia guilliermondii	Bonatsou et al. (2015)
Wickeramomyces anomalus	Bonatsou et al. (2015)
Dekkera, Galactomyces, Hansenula, Brettanomyces, Rhodotorula, Torulopsis, Hanseniaspora, Hyphopichia, Trichosporon, Yarrowia, Saccharomyces, Saccharomycopsis, Schizosaccharomyces, Sporobolomyces, Torulaspora, Geotrichium isatchenkia, Kazachstania, and *Zygosaccharomyces*	El Sheikha and Montet (2014), Lv et al. (2013), Omemu et al. (2007), Tamang and Fleet (2009), Watanabe et al. (2008), Wouters et al. (2002)

3.4.2 Kefir

Kefir, yeast containing fermented milk, serves as one of the great sources of probiotic yeast strains. It is a fermented, creamy milk product with little acidity. A consortium of yeast and bacterial strains (10^6–10^8 CFU/g), stick to a polysaccharide matrix makes up its starter culture (Prado et al., 2015). Around 23 yeast species, particularly *S. cerevisiae* and *K. marxianus* have been reported to be presented in kefir. Recently, kefir grains have been reported to have both culturable and non-culturable yeast species in them, predominantly the strains belonging to *Saccharomyces*, *Torula*, *Candida*, *Kluyveromyces*, *Pichia*, *Kazachatania*, and *Zygosaharomyces* (Diosma et al., 2014; Leite et al., 2012; Magalhães et al., 2011; Miguel et al., 2013). Like koumiss, kefir is also produced by acid-alcoholic fermentation of milk by microbes present in kefir grains (Kesenka et al., 2017). Various yeast, acetic acid, and lactic acid bacterial strains present in kefir grains are responsible for this acid-alcoholic fermentation. In addition to anticarcinogenic, antiinflammatory, antihypertensive, antidiabetic, and probiotic effects, kefir also has pleasing organoleptic features which ultimately has made kefir a focus of interest in recent years (Guzel-Seydim et al., 2011; Leite et al., 2013; Nielsen et al., 2014; Rosa et al., 2017). Kefir is also reported to have shown antifungal properties against *Aspergillus flavus* by inhibiting its growth. Owing to its favorable organoleptic and probiotic properties, many nations like Malaysia have started the manufacture and commercialization of this fermented milk.

3.4.3 Leben

It is kefir-like fermented milk products from Arab countries and is prepared by inoculating a mesophilic LAB, thermophilic culture of yogurt, and yeasts in fresh milk. Among yeast species, *K. marxianus* is a dominant strain found in Leben. Due to the presence of yeast, ethanol, acetoin, and diacetyl are produced in Leben.

3.4.4 Liqvan (Lighvan/Levan)

It is a variety of Iranian raw milk cheese, made from unpasteurized and unprocessed ewe's milk in Liqvan village of Iran. The production process of Liqvan starts within 2 hours of milking and it depends upon the indigenous microflora (fungi and bacteria) of milk for coagulation. The ripening process, which takes around 6 months, is carried out either in natural caves or in man-made holes. It is believed that the indigenous fungal (yeast) microbiota of ewe's milk is responsible not just for the production of Liqvan cheese, but also for its peculiar flavor and texture. Therefore, the yeast profile of Liqvan cheese has been studied by many researchers, to identify the dominant fungal strains in each step of cheese production. *Pichia fermentans, Candida pararogosa, Candida zeylanoides, Geotrichum candidum, Cladosporium ramotenellum*, and *Cryptococcus magnus* are found to be the active yeast strains, mainly responsible for converting step of milk to curd. The yeast strains which modify the prepared curd to cheese are *Candida sake, Debaryomyces hansenii, Kluyveromyces lactis, S. cerevisiae, P. fermentans*, and *C. zeylanoides* (Ramezani et al., 2018).

Although yeasts are not directly involved in cheese production, but they are sometimes utilized as secondary cultures, where they add significant aroma and texture. *D. hansenii* and *Yarrowia lipolytica* are widely used for achieving aromatic essence in Muenster and parmesan cheeses. In surface-ripened cheeses (smear-ripened) yeasts play a prominent part in enhancing the survival of LAB, *Brevibacterium linens*, *Micrococci*, and *Microbacterium lacticum* by secreting vitamins and amino acids, by enhancing the lowered pH through proteolysis as well as by the production of alkaline products. In gorgonzola and other similar cheese varieties, yeast gives a desirable open doughy structure through gas production. Through proteolysis and lipolysis yeasts produce several aromatic compounds and by hydrolyzing specific casein fractions, a positive effect on the growth of LAB and *Penicillium roquefortii* is being imparted in blue-veined cheeses. An acidic, cheesy, and fruity aroma is being produced by *D. hansenii* during the ripening of aseptic curd slurries.

3.5 Mold: A brief overview of molds and their role in dairy fermentation

Molds are an indispensable part of the fermented food industry. They are aggressively employed in food fermentations, as molds can either be used to add proteins, fiber, vitamins in the product, or can be consumed as single-cell proteins (SCP) (Nout, 2007). In the dairy sector, molds have a limited but noteworthy role to play and current research is going on to explore mold's metabolic activities in dairy food fermentations.

3.5.1 Viili

It is a well known set curd-like fermented milk product of Finland. The starter culture used for fermentation is a mesophilic LAB culture along with *Galactomyces geotrichum* mold. Viili has white to light yellow colored velvety, matte surface owing to the growth of strictly oxidative mold culture on the surface of the product. *G. geotrichum* strains having minimum lipase activity are preferred for viili production to prevent autooxidation of fats. Along with this, the mold develops a particular aroma and prevents contamination of the product by wild mold strains.

3.5.2 Norwegian tettemelk and Swedish långfil

These are similar products where *G. geotrichum* and similar strains provide a nut-like flavor in the product.

3.5.3 Role in ripening of cheese

Mold ripened cheeses can be categorized into internally and externally ripened cheeses depending on the access of mold to the cheese. Blue-veined, Danish blue, Gorgonzola, Roquefort, and Stilton are some popular varieties of internally ripened cheese while Brie, Camembert, Carrel de est are externally ripened cheese varieties.

3.5.3.1 Fusarium domesticum

It has been utilized for cheese smear (fermentation on the cheese surface) for many years. Another strain, *Fusarium solani* DSM 62416 has also been isolated from Vacherin cheese (Thrane, 2007).

3.5.3.2 *Penicillium camemberti*

It is the popular name of mold used thoroughly in the production of white mold cheeses (semi-soft cheeses) (Frisvad & Samson, 2004). A fine mist of *Penicillium camemberti conidia* is sprayed over the surface of young cheese curd, which after incubation imparts a thin whitish-grey rind to the cheese. Although some other species of this genera like *Penicillium commune, Penicillium biforme, Penicillium fuscoglaucum*, and *Penicillium palitans* are also found on cheese as green cheese mold, they are not suitable for cheese fermentation. However, *P. commune* is found to be the wild type ancestor of *P. camemberti* (Giraud et al., 2010; Pitt et al., 1986; Polonelli et al., 1987). Important mycotoxins secreted by *Penicillium* sp. can also be detected in white mold cheeses. The mycotoxins secreted are identified as cyclopiazonic acid and rugulovasine A and B (Frisvad & Samson, 2004), and cyclopiazonic acid (Bars et al., 1988; Le Bars, 1979; Teuber & Engel, 1983). Among white cheese molds, *Penicillium caseifulvum*, a closely related species to *P. camemberti*, has an added advantage in not secreting cyclopiazonic acid (a mycotoxin), which is generally produced by *P. camemberti* (Frisvad & Samson, 2004; Lund et al., 1998).

3.5.3.3 *Penicillium roqueforti*

It is another famous name of the mold widely used in the production of blue-veined cheeses, which is a semi-soft cheese ripened by the growth of *P. roqueforti*. Some popular varieties of blue-veined cheeses include Roquefort, Gorgonzola, Stilton, and Danish blue. The Cabrales and Kopanisti cheese of Spain and Greece, respectively may also be included in this group. After the salting of cheese curd, a network of airy pockets is created by piercing the curd with a needle-like equipment. These pockets then allow the gaseous exchange in cheese and promote the veins of mold to grow vigorously throughout the cheese's structure. However, some closely related species such as *Penicillium carneum, Penicillium paneum* or *Penicillium psychrosexualis* are mis-referred to as *P. roqueforti* and the former three species produce several mycotoxins. *P. roqueforti* in pure culture itself produces several secondary metabolites such as andrastin A, PR-toxin, mycophenolic acid and roquefortine C (Frisvad et al., 2004; Nielsen et al., 2005). Among these mycotoxins produced by *P. roqueforti*, PR-toxin is unstable in cheese and is converted to quite less harmful PR-imine (Engel & Prokopek, 1979). The rest of the three mycotoxins produced by *P. roqueforti*, have been reported to have minor consequences on human health (Fernández-Bodega et al., 2009; Larsen et al., 2002).

3.5.3.4 *Penicillium nalgiovense*

It was originally isolated from cheeses of Nalzovy, but presently it is not commonly used for fermenting cheeses. *Verticillium lecanii*, which has been changed to *Lecanicillium lecanii* (Zare & Gams, 2001), has been listed as a potentially useful strain for cheese ripening processes.

3.6 Exploration of probiotic potential of fungal cultures

Probiotics find its origin from the Greek word *probios*, means "for life," and as per the Food and Agriculture Organization (FAO)/World Health Organization (WHO), it is described as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO, 2001). Various organisms, like yeast, bacteria, and mold, are used as probiotics. Amongst the bacteria, *Lactobacillus* sp. and *Bifidobacterium* are prevalently used as probiotics. An efficient probiotic organism should demonstrate the following desirable features: safety, viability, and sustenance in gastrointestinal tract (Mathipa & Thantsha, 2017). Additionally, probiotic strains must have the potential to bind to intestinal epithelial cells besides possessing antimicrobial activity (Shokryazdan et al., 2017). It is known that the intake of probiotics is considered to contribute to enhancing and sustaining harmonious gut microbiota. Other prominent characteristic activities of probiotics include antidiabetic (Yadav et al., 2007), antiallergic (Masuda et al., 2012), cholesterol-lowering (Anandharaj & Sivasankari, 2014), antipathogenicity (Kumar et al., 2018; Zhou et al., 2016), antiobesity (Rouxinol-Dias et al., 2016), antiinflammatory (Junjua et al., 2016), anticancer (Sharma et al., 2018), and antihypertensive (Ahtesh et al., 2018). In terms of market trends, the fungi fair better as probiotics organisms than the bacterial probiotics, owing to their unique cellular architecture. The cell envelope is composed of an outer layer made up of mannans and an inner layer consisting of chitin and 1,3- and 1,6-β-glucan (Lipke & Ovalle, 1998), which permits a smooth shift through the gastrointestinal environment. Many fungal genera have been reported to belong to the probiotic family: *Candida humilis, D. hansenii, Debaryomyces occidentalis, K. lactis, Kluyveromyces lodderae, Kluyveromyces marxiamus, Pichia kluyveri, S. cerevisiae* var. *boulardii, Pichia kudriavzevii, Torulaspora delbrueckii, Issatchenkia orientalis, Candida tropicalis, Candida saitoana, Candida pintolopesii, Cryptococcus albidus, Meyerozyma caribbica, Wickerhamomyces anomalus*, and *Candida famata* (Amorim et al., 2018; Arevalo-Villena et al.,

2017; Cho et al., 2018; El-Baz et al., 2018; Greppi et al., 2017; Maccaferri et al., 2012; Ochangco et al., 2016; Puppala et al., 2019; Smith et al., 2014; Srinivas et al., 2017). *S. cerevisiae* var. *boulardii* or *S. cerevisiae* Hansen CBS 5926, is the best-studied yeast to date. The yeast species is widely endorsed to treat acute gastrointestinal problems like inflammatory bowel disease (Kelesidis & Pothoulakis, 2012; Madsen, 2001). The fermented food or drink often contain prospective probiotic yeasts and kefir is one of the fine examples of a fermented milk product. The starter culture required for the production of kefir consists of a concoction of yeast and bacterial strains (10^6-10^8 CFU/g) (Prado et al., 2015). Lourens-Hattingh and Viljoen (2001) studied the growth and survivability of probiotic yeast in bio-yogurt, ultra-high temperature (UHT) yogurt, and milk. The addition of these dairy products with *S. boulardii* resulted in maximum counts surpassing 10^7 CFU/g over 4 weeks at 4°C. The yeast count was found to be significantly more in the fruit enriched yogurt, because it's being enriched in fruit-based sugars. Azhar and Abdul Munaim (2019) reported the isolation of probiotic strains M1 (*S. boulardii*), M3 (*Kazachstania unispora*), and A1 (*Kodamaea ohmeri*) from the Malaysian kefir drink, possessing the potential to withstand the acidic environment of the gastric tract and exhibits a prolific growth profile at human body temperature. The organic acid, bacteriocins, antibiotic factors, and H_2O_2 produced during the growth phase of yeast confer the antimicrobial potential (Hatoum et al., 2012; Yadav & Shukla, 2017). A study by Saleh et al. (2014) revealed that the administration of *Aspergillus awamori* resulted in an increase in body weight over the control group fed with a basal diet. In another study by Sugiharto et al. (2018), intake of *Chrysonilia crassa* by broiler chickens presented a significant increase in growth and immune responses, albeit less than the *Bacillus subtilis* based conventional feed additives. A study by Gil de los Santos et al. (2018) on broiler chickens established the potency of *Pichia pastoris* and *S. boulardii* in strengthening feed qualities. Although *S. cerevisiae* has been researched widely because of its probiotic ability, relatively little emphasis is laid on its genetic transformation. Interestingly, *S. boulardii* is targeted in all those works. Genetically engineered probiotic *S. cerevisiae* strain can express proteins of interest that ward off pathogenic microorganisms. These strains have a synergistic combination of probiotic and therapeutic effects and constitute better carriers of therapeutic protein than probiotic bacteria. Owing to specific pathogen-related molecular patterns (PAMP) on their surface, various probiotic bacteria induce varied immune responses (Owen et al., 2013), but may result in multidrug-resistant species of bacteria due to exchange of antibiotic resistance gene with the pathogenic bacteria (Cummins & Ho, 2005; Czerucka et al., 2007; Mathur & Singh, 2005). *S. boulardii* is a well-established probiotic strain, whose effectiveness has been documented against several gastrointestinal disorders (Guslandi et al., 2003), antibiotic-associated diarrhea (Kotowska et al., 2005), *C. difficile* infections (McFarland, 2009), Crohn's disease (McFarland, 2010), and gut inflammatory expositions in AIDS patients (Villar-García et al., 2015). One striking benefit of *S. boulardii* in contrast to prokaryotic probiotics is the presence of post-translational modification (Tokmakov et al., 2012). Genetic engineering is an effective tool to augment the intrinsic benefits of *S. boulardii* or to introduce new probiotic properties to it. The transformation of *S. boulardii* MYA- 796 with pYC440, resulted in the generation of hygromycin B resistant mutant. *S. boulardii* uracil auxotrophs were obtained through classical UV mutagenesis approach by mutagenizing wild type stain lyo (Hamedi et al., 2013). The further transformation of ura3 − *S. boulardii* was carried out with plasmid pGEM-Teasy, thus reinstating its uracil production potential. The transformed strain was found to endure low pH present in the GI tract, besides retaining its probiotic abilities under in vitro assays conditions. In another independent work, *S. boulardii* strain MYA-797were found to grow under anaerobic conditions, as found in GIT (Hudson et al., 2014). The strain was further transformed with a GFP sequence encoding plasmid, and administered orally to the mice. The GFP-expressing ura3 − *S. boulardii* cells were recovered from mice Peyer's patches, and lymphoid organs present in the small intestine. The transformed *S. boulardii* neither show any adverse side effect nor resulted in loss of probiotic benefits when administered to animal models (Hudson et al., 2014; Michael et al., 2013). Various techniques of transformation were developed and widely been used in *S. cerevisiae*, namely spheroplast, electroporation, biolistic, *Agrobacterium tumefaciens*-mediated, and glass beads techniques (Kawai et al., 2010). More recently, CRISPR-Cas9 genome engineering approach is used by researchers to generate His, Tryp, Leu, and Ura auxotrophs (Liu et al., 2016; Stovicek et al., 2017). The CRISPR-Cas9 system has substantially higher performance, flexibility, and sturdiness in eukaryotic environments than the conventional approaches. Si et al. (2017) successfully constructed yeast strains with more than 90% of the genes overexpressed or knockdown mutants using one-step automated pathway. The method led to the enhancement of editing efficiency utilizing the *Streptococcus pyogenes* CRISPR-Cas9 complex leading to breaks in the double strands of genome, thus resulting in homologous recombination or non-homologous end-joining. The oral administration of Ura auxotroph of *S. boulardii* expressing OVA-CPE (union of ovalbumin and enterotoxin), resulted in enhanced secretion of serum IgG and fecal IgA (Bagherpour et al., 2018; Koch et al., 1996; Suzuki et al., 2012). Hou et al. (2018) developed a tool using a DNA cassette called WICKET, which entails universal homology arms flanking a Cas9 target site. When several copies of WICKET are inserted into the genome using CRISPR-Cas9, the entire exogenous gene pathway is accepted after Cas

9 produces a double-strand break. The authors could incorporate a β-carotene pathway into the *S. cerevisiae* using this method that paves the way to further similar pathways for genomic integration. With the help of pooled-segregant whole-genome sequence analysis with *S. boulardii* and *S. cerevisiae* parent strains the underlying QTLs were mapped and mutant alleles of SDH1 and WHI2 were identified as the causative alleles by Offei et al. (2019). Both genes contain a single nucleotide polymorphisms (SNP) unique to *S. boulardii* (sdh^{1F317Y} and whi^{2S287*}) generating large levels of acetic acid that gives the first molecular explanation of *S. boulardii* exerting probiotic action.

3.7 Molecular approaches to study fungal dairy fermented foods

Molecular biology advances have provided more knowledge to scientists about food-associated micro-organisms and have also provided the ability for the detection, identification, and typing of food-based microorganisms in an accurate and efficient manner. The dairy microbiologists are involved primarily in studying the varied types of microbes in dairy processes and in correlating the presence of such microbial species and strains with the desirable fragrance and sensory properties of the products. The issues with traditional detection approaches make it impossible for them to accurately classify or use microbial taxa biochemically from food, even if relying on the portable kits. Therefore, substantial research work has been in progress to develop alternative identification techniques, integrating swiftness, consistency, and cost-effectiveness. Methods based on molecular traits instead of phenotypical traits meet these requirements. The techniques such as PCR-DGGE/TGGE/single-strand conformations (SSCP) are used for the identification of microorganisms at the genus and species level without the need of culturing of microorganisms. Polymerase chain reaction (PCR) amplification is needed afterward, and the DNA encoding for ribosomal RNA is the most widely used target of identification at the species level. The fingerprint consists of many bands that relate to as many microbial organisms and represents the identity of starter culture. Every species will then eventually be identified by purifying and sequencing the band and comparing it to the NCBI Gene Bank repositories. Such approaches have the advantage, that a microbiota at the species level is identified and monitored without being isolated on culture media. Instead of isolation, DNA can be extracted directly from the microorganisms present in the medium. Rychlik et al. (2017) established the presence of *Candida incospicua, G. geotrichum*, *K. marxianus, P. kudriavzevii*, and *Trichosporon* sp. using the PCR- DGGE technique in Wielkopolska fried cheese. Ramezani et al. (2018) explored the fungal population during cheese manufacturing by denaturing gradient gel electrophoresis (DGGE) analysis, targeting PCR amplicons of the D1 region of the 26S rRNA gene., Although the DGGE profiles of yeast amplicon showed different results during the ripening process from the extracted DNA and RNA, the core group found to be present in all stages of the ripening process was *Candida* although *Kluyveromyces, Pichia, Galactomyces, Saccharomyces*, and *Cryptococcus* were also abundantly present. The diversity profiling of microorganisms in the final products and during processing steps have been achieved by PCR-based approaches, that is, PCR-DGGE, PCR-TTGE, PCR-RFLP, RAPD-PCR, rep-PCR, length heterogeneity (LH-PCR), automated ribosomal intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism (SSCP-PCR), quantitative PCR (qPCR). Callon, Delbès, Frédérique & Montel (2006) profiled *K. lactis*, *K. marxianus*, *C. zeylanoides*, *D. hansenii* and *S. cerevisiae* in Salers cheese using the PCR-SSCP approach. whereby, primers for yeast were designed for the amplification of V4 region of the 18S rRNA gene. The yeast species were observed to be *K. lactis, K. marxianus, C. zeylanoides, D. hansenii* and *S. cerevisiae*. PCR-DGGE fingerprinting is also used to identify the microbiological qualities of food. The technique of fluorescence in situ hybridization (FISH) using 16S rRNA probes is a viable tool to study the spatial dispersion of the bacteria inside the cheese matrix (Bottari et al., 2006). Mounier et al. (2009) designed the FISH probes of *Candida catenulata*, *Candida intermedia*, *Geotrichum* sp., and *Y. lipolytica* for their direct detection in Livarot cheese. While the classical phenotype approach (biotyping) is still very important for everyday studies, genotypical approaches have contributed more to the profound characterization and differentiation of microorganisms. The most common typing techniques used to characterize milk products microflora are: random amplification of polymorphic DNA (RAPD)-PCR and related approaches, such as BOX-PCR, Amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (APPCR), restriction endonuclease analysis, and pulsed-field gel electrophoresis (REA-PFGE); repetitive element sequence-based PCR (REP-PCR), and sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell polypeptides (WCPs). Such methods are used in conjunction or alone.

3.8 Designing a novel starter

Starting culture is an active microbial preparation, which is added intentionally to initiate desirable changes during fermented product development (Hati et al., 2013). Starter cultures play a significant industrial role in the processing,

development of taste, and texture of fermented products. (Cogan et al., 2007). There is a persistent need for the creation of novel starter cultures for the development of new commercial products along with greater characterization to guarantee safe and functional products. Many factors directly impact, positively or negatively, the selection of starter culture for dairy fermentation such as history of safe use, production of undesirable metabolites, and competence against pathogens. Within the dairy industry, the mycelial fungal cultures, *P. roqueforti* and *P. camemberti* have been intensely studied for their proteolytic and lipolytic activities with the help of techniques such as mass spectroscopy. The analysis of functional metabolic characteristics may help in efficient strain selection. *P. roqueforti* produces two or more extracellular lipases and an intracellulose lipase, all of which are involved in lipid hydrolysis into free fatty acids (FFA), which constitute principal flavors of cheese as well as precursors for other aroma substances, such as methyl-ketones (Gillot et al., 2016). The functional diversity of 55 representative strains of *P. roqueforti* for the cheese production were screened for proteolytic activity by determining free NH_2 amino groups, aroma compounds using HS-Trap gas chromatography-mass spectrometry (GCMS), and mycotoxin production using HS-Trap GCMS and mycotoxins via accurate-mass quadrupole time-of-flight (Q-TOF) system (Gillot et al., 2016). An essential criterion for fungus to be used as a starter culture is dependent on the fact that there are no undesirable secondary metabolites e.g., mycotoxins produced by them. An ideal fungal starter culture is one that does not produce any such metabolites (Geisen & Farber, 2001). The genome of fungal starter cultures has been studied to identify and understand the regulation of gene encoding technological properties and undesirable properties such as mycotoxin production (Esser & Bennett, 2002). A gene technological approach called "gene disruption" is followed that inactivate the unwanted gene in an organism specifically. Unlike a gene inactivation with a chemical mutagen or radiation, this approach inactivates only the target gene that makes it of special interest in the optimization of strains already in use. There can be no secondary mutations that can adversely impact a starter culture's activity with this approach (Geisen & Farber, 2001). The approach was adopted for the transformation of *Penicillium nalgiovense* to a non-penicillinogenic strain (Geisen & Leistner, 1989). The fungal starter cultures must also contribute to the microbiological safety of a product by resisting the growth of pathogenic microbes. Several antagonistic proteins with GRAS status (generally recognized as safe) namely bacteriocin, lysozyme, or glucose oxidase exist. With the help of *P. nalgiovense* transformation system (Geisen & Leistner, 1989) the glucose oxidase (*god)* gene from *Aspergillus niger* was introduced into *P. nalgiovense*, which has a weak endogenous *god* gene expression. The transformed strains were able to inhibit indicator organisms *Staphylococcus aureus*, *Listeria monocytogenes*, and *Staphylococcus enteritidis* (Geisen & Farber, 2001).

3.9 Conclusion and perspective

The fungi have tremendous potential in the dairy industry. A wide variety of fungal fermented dairy foods are presented in the chapter. The advancements in the field of molecular biology have resulted in the development of comprehensive, consistent, and efficient approaches for detection, identification, and improvement of fungal strains essential for dairy fermentation. Future research in the field will further pave the path to understand the show the mechanisms of modified fungal cultures at cellular and molecular levels in humans. Next-generation DNA sequencing such as pyrosequencing can prove to be a powerful technique. The technique will not eliminate the need for labor-intensive cloning, but will also provide knowledge about the molecular aspects of flavor and taste of fermented dairy foods. Also, research focusing on proteomic and metabolomic approaches of fungal cultures will be essential.

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Chapter 4

Recent trends in alkaline fermented foods

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4.1 Introduction

During fermentation, microorganisms transform the chemical constituent of raw material thereby improving its nutritional value. The consumption of fermented foods provide health-promoting bioactive compounds to consumers. Several traditional methods are prevalent worldwide for the production of fermented foods and seasoning agents. There are four basic types of fermentation processes: acetic acid fermentation, alcoholic fermentation, lactic acid fermentation, and alkaline fermentation (Anal, 2019; Blandino et al., 2003; Mishra et al., 2017). These processes differ in microorganisms, raw material, and fermentation conditions (Anal, 2019; Nwachukwu et al., 2010). In alcoholic fermentation, yeast species result in the production of ethanol as an end product. In acetic acid fermentation, the acetic acid bacteria transforms the alcohol to acetic acid, for example, the production of vinegar. In lactic acid fermentation, the lactic acid bacteria (LAB) produces organic acid and various other compounds in food material. Alkaline fermentation involves the fermentation of raw materials having high protein content like legumes, oilseeds and fish to produce condiments. In this type of fermentation, the metabolic processes revolve around the degradation of protein to peptides, amino acids and ammonia thereby raising the pH to 8 or above (Aniche et al., 1993; Omafuvbe et al., 2000; Sarkar & Tamang, 1995). In alkaline fermentation *Bacillus* species are the main microorganisms responsible for the breakdown of proteins to simpler constituents. This provides easily digestible protein with low fat to consumers. Indigenous alkaline foods are relished throughout the world due to their peculiar aroma. The addition of these fermented foods and condiments enhances the taste of the food to the next level (Mishra et al., 2017). In addition to this, these foods are high in easily digestible nutrients and have a prolonged shelf life giving a sense of food security to locals. Moreover, most of the substrates used in alkaline fermentation contain many antinutrients and toxins which become suitable for consumption only after their detoxification during fermentation. It has been found that the concentration of various amino acids like methionine, cysteine, tyrosine, leucine, isoleucine, phenylalanine and lysine increases in the food after fermentation. In Africa and Asia, alkaline fermentation is very common and alkaline fermented condiments are an inseparable part of the diet in the native communities (Dakwa et al., 2005; Odunfa, 1988; Okpara & Ugwuanyi, 2017; Olusupe & Okorie, 2019; Parkouda et al., 2009; Villéger et al., 2017). In the majority of these countries, people use starchy staples like cereals, yam cassava, and plantain for the fermentation. All these foods are mostly rich in carbohydrates, but poor in nutrients. Seeds used for fermentation are from cultivated plants (Inatsu et al., 2006; Terlabie et al., 2006) as well as from many wild trees (Achi, 1992; Ejiofor et al., 1987; Ogunshe et al., 2007; Ouoba et al., 2004). Recently there has been a marked improvement in the interest and research into alkaline fermentation (Dirar, 1993; Steinkraus, 2004). There are several alkaline fermented foods which are admired by the people in Africa and Asia (Tables 4.1 and 4.2).

4.2 Alkaline fermented foods of Africa

4.2.1 Dawadawa

In the production of Dawadawa, seeds of the African locust (*Parkia biglobosa*) bean tree are used as starting raw material (Azokpota et al., 2006; Omafuvbe et al., 2004). Its production is time-consuming and laborious. Various processing parameters like duration of fermentation, type of microbial species, type of substrate used varies from one region to another in Africa (Diawara et al., 1992; Sanni, 1993). For the preparation of Dawadawa (Fig. 4.1), the seeds are cleaned and cooked for 12−24 hours until soft. The seeds are then washed and dehulled. The cotyledons are then packed in jute

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TABLE 4.1 Raw material and conditions required for the production of tungrymbai, bekang and peruyaan.

	Tungrymbai	Bekang	Peruyaan
Raw material	Soybean	Soybean	Soybean
Place	Khasi and garo in Meghalaya	Mizo in Mizoram	Apatani tribes in Arunachal Pradesh
Duration of soaking	4–6 h	10–12 h	Only washed
Duration of cooking	1–2 h	2–3 h	2–3 h
Leaves used	Fresh leaves of *Clinogyne dichotoma* (lamet)	Fresh leaves of *Calliparpa aroria* (nuhlhan) or leaves of *Phrynium* sp. (hhahnial)	Leaves of ginger
Fermentation	3–5 days	3–4 days	3–5 days
Microorganism	*Bacillus subtilis*, *B. licheniformis*, *B. pumilus*	*Bacillus subtilis*, *B. licheniformes*, *B. pumilus*, *B. circulans*	*B. subtilis*, *B. amyloliquefaciens*, *Vagococcus lutrae*, *Pediococcus acidilactici*, *Enterococcus*

Modified from : Tamang, J. P. (2015). Naturally fermented ethnic soybean foods of India. *Journal of Ethnic Foods*, *2*(1), 8–17. doi:10.1016/j.jef.2015.02.003 (Tamang, 2015).

sacks or banana leaves or put in earthenware pots for fermentation. The duration of fermentation varies depending upon the local need. It is 18 hours for afitin, 48 hours or more in Nigerian dawadawa and about 72 hours for netetu in Senegalese (Azokpota et al., 2006; Ndir et al., 1994; Odunfa, 1988). Many workers have tried to modify its production process to make it less laborious (Achi, 2005; Alabi et al., 2005; Odunfa, 1988). Modified procedures have reduced the processing time and energy requirement (Fig. 4.2), and has also attracted the attention of many entrepreneurs (Blandino et al., 2003). In many parts of Africa, dawadawa cubes are now sold with improved packaging under different brand names (Alabi et al., 2005). *Bacillus subtilis* is reported as the dominant microorganism in dawadawa samples (Odunfa & Oyewole, 1986). In addition to this *B. pumilus*, *B. licheniformes*, *Leuconostoc dextranicus*, *L. mesenteroides*, *Micrococcus* spp. and *Staphylococcus* spp. are also responsible for fermentation. (Ndir et al., 1997).

4.2.2 Soumbala

Soumbala is also an alkaline fermented product of seeds of African locust (*P. biglobosa*) by *Bacillus* spp. It is a popular traditional condiment in Burkina Faso. Microorganisms responsible for its production are *B. subtilis*, *B. pumilus*, *B. cereus* and *Brevibacillus borstelensis* (Ouoba et al., 2004). The traditional process of preparation is also similar to dawadawa with slight variations (Fig. 4.3).

4.2.3 Okpeye

Okpeye is prepared by the fermentation of seeds of Mesquite (*Prosopis africana*). Its traditional methods of production vary among different cultures (Achi, 1992; Oguntoyinbo et al., 2007; Omafuvbe et al., 1999). In the production of Okpeye (Fig. 4.4), seeds are boiled for 15–24 hours. Dehulling of seeds is done by pressing them between palms. Cotyledons thus obtained are washed and dehydrated in a pot lined with akwukwo okpeye (*Alchornea cordifolia*) leaves. In the absence of this leaf, banana leaves are also used by locals. If not dry heated they are boiled again for 3–5 hours. The cotyledons are then transferred to a basket or a tray lined with leaves of akwukwo okpeye. The cotyledons are then covered again with the leaves and kept in sunlight for fermentation for 3–5 days. During the night they are shifted inside the house to avoid precipitation. Fermentation takes place at an uncontrolled temperature. After fermentation the cotyledons become dark brown having a characteristic ammonical smell. The fermented product obtained is ground to make a paste and molded in different sizes and sundried for different lengths of time. (Okpara & Ugwuanyi, 2017). The dried condiment can be stored for a long time with occasional sun drying. Some species of *Bacillus* like *B. subtilis*, *B. puhilus*, *B. licheniformis* and *B. megaterium* are involved in the process of fermentation. In

TABLE 4.2 Alkaline fermented foods from Africa.

Raw material	Product name	Region	Microorganism	References
African locust bean (*Parkia biglobosa*)	Dawadawa (Iru)	Nigeria	*B. subtilis*, *B. brevis*, *B. pumilus*, *B. licheniformis B. megaterium*, *B. polymyxa Staphylococcus* spp., *Leuconostoc* spp., *Pseudomonas aeruginosa*	Odunfa (1981), Odunfa and Oyewole (1986), Jideani and Okeke (1991), Omafuvbe et al. (2004), Sanni et al. (2000)
	Soumbala	Burkina Faso	*B. subtilis*, *B. thuringiensis B. pumilus*, *B. cereus*, *B. badius*, *B. sphaericus*, *B. licheniformis*, *Paenibacillus alvei*, *P. larvae*, *Brevibacillus borstelensis*, *B. mycoides*, *B. laterosporus*	Sarkar et al. (2002), Ouoba et al. (2004)
	Netetu	Senegal	*B. subtilis*, *B. licheniformis*, *B. coagulans*, *B. pumilus*, *Micrococcus* spp., *Staphylococcus* spp.	Ndir et al. (1994, 1997)
	Afitin/Sonru	Benin	*B. subtilis*, *B. cereus*, *B. licheniformis*, *Staphylococcus* spp.	Azokpota et al. (2006)
Mesquite (*Prosopis africana*)	Okpehe	Nigeria	*B. subtilis*, *B. megaterium*, *B. cereus*, *B. pumilus*, *B. licheniformis*, *Micrococcus luteus*, *Staphylococcus epidermidis*, *Proteus* spp. *Enterobacter cloacae*, *Escherichia coli*, *Lactobacillus* spp., *Klebsiella pneumoniae*, *Pseudomonas* spp., *Enterococcus* spp.,	Achi (1992), Ogunshe et al. (2007), Oguntoyinbo et al. (2007), Omafuvbe et al. (1999)
Castor oil bean (*Riccinus communis*) Melon (*Citrullus vulgaris*)	Ogiri	Nigeria	*B. subtilis*, *B. metaterium*, *B. licheniformis*, *P. aeruginosa* *Staphylococcus* spp.	Jideani and Okeke (1991)
African oil bean (*Pentaclethra macrophylla*)	Ugba/Ukpaka	Nigeria	*B. subtilis*, *B. brevis B. pumilus*, *B. megaterium*, *B. licheniformis*, *B. cereus B. polymyxa*, *B. coagulans*, *B. macerans*, *Lactobacillus* spp., *Micrococcus roseus*, *Staphylococci saprophyticus*, *Pseudomonas chlororaphis*	Isu and Njoku (1997), Isu and Ofuya (2000), Mbajunwa et al. (1998), Sanni et al. (2000, 2002)
Saman tree (*Albizia saman*)	Aisa	Nigeria	*B. subtilis*, *B. megaterium*, *B. cereus* var. *mycoides*, *B. pumilus*, *B. licheniformis*, *B. coagulans*, *B. pumilus Staphylococcus saprophyticus. S. aureus*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Escherichia coli*	Ogunshe et al. (2006)
Cotton seed (*Gossypium hirsutum*)	Owoh	Nigeria	*B. subtilis*, *B. polmyxa*, *B. pumilus*, *B. megaterium*, *B. licheniformis*, *B. brevis*, *P. aeruginosa*, *Staphylococcus* spp.	Jideani and Okeke (1991), Omafuvbe et al. (2004), Sanni et al. (2000), Sanni and Ogbonna (1991)
African yam bean (*Sphenostylis stenocarpa*)	Owoh	Nigeria	*B. subtilis*, *B. pumilus*, *B. licheniformis*, *Staphylococcus* spp.	Ogbonna et al. (2001)
Roselle (*Hibiscus sabdariffa*)	Bikalga	Burkina Faso	*B. subtilis*, *B. cereus*, *licheniformis*, *B. pumilus*, *B. badius*, *Brevibacillus bortelensis*, *B. sphaericus*, *B. fusiformis*, *Staphylococcus* spp.	Bengaly and Etude (2001), Ouoba et al. (2008)
Glycine max Soybean	Soydawadawa	Ghana	*B. subtilis*, *B. cereus*, *B. pumilus*, *B. firmus*, *B. licheniformis*	Dakwa et al. (2005)
Bambara groundnut (*Vigna subterranea*)	Dawadawa type product	Nigeria	*B. subtilis*, *B. licheniformis*	Amadi et al. (1999)

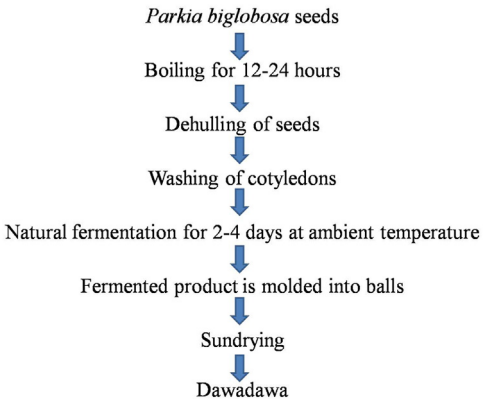

FIGURE 4.1 Traditional method of production of dawadawa. *Modified from Azokpota, P., Hounhouigan, D.J., & Nago, M.C. (2006). Microbiological and chemical changes during the fermentation of African locust bean (*Parkia biglobosa*) to produce afitin, iru and sonru, three traditional condiments produced in Benin.* International Journal of Food Microbiology, 107*(3), 304–309. doi:10.1016/j.ijfoodmicro.2005.10.026; Omafuvbe, B.O., Falade, O.S., Osuntogun, B.A., & Adewusi, S.R.A. (2004). Chemical and biochemical changes in African Locust Bean (*Parkia biglobosa*) and Melon (*Citrullus vulgaris*) seeds during fermentation to condiments.* Pakistan Journal of Nutrition, *140–145. doi:10.3923/pjn.2004.140.145.*

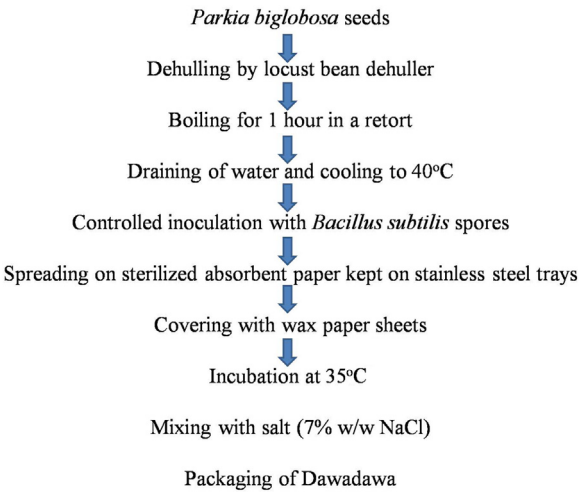

FIGURE 4.2 Modern production of dawadawa. *Modified from Achi, O.K. (2005). Traditional fermented protein condiments in Nigeria.* African Journal of Biotechnology, 4*(13), 1612–1621. http://www.academicjournals.org/AJB/PDF/Pdf2005/Spe%20Rev/Achi.pdf; Olusupe et al. (2019).*

addition to this *Micrococcus* spp., *Klebsiella pneumonia*, *Enterobacter cloacae*, *Staphylococcus epidermis*, *Lactobacillus* spp., *Pseudomonas* spp. and *Proteus* spp., have also been isolated during fermentation (Ogunshe et al., 2007; Omafuvbe et al., 1999).

4.2.4 Ogiri

Ogiri is the alkaline fermented food condiment prepared from seeds of castor oil seeds(*Ricinus communis*), alternatively, it is also prepared from the seeds of melon (*Citrullus vulgaris*) and fluted pumpkin seeds (*Telfairia occidentalis*). It is used for the preparation of soups by an ethnic group, Igbo in Southeastern Nigeria. For the preparation of Ogiri (Fig. 4.5), the castor seeds are dehulled manually and enveloped in blanched plantain or banana leaves and then boiled for 6–8 hours. The seeds are then fermented for 4–6 days near a fireplace. After fermentation, cotyledons are sticky with a characteristic aroma. It is then ground using mortar and pestle into a fine paste, small portions of paste is then wrapped in banana leaves and kept near the fireplace for further fermentation or maturation for 2–3 days. The pH of the product at the end of fermentation is found to be around 7.9 (Omafuvbe et al., 2004). The microorganisms responsible for fermentation are *B. subtilis*, *B. pumulis*, and *B. licheniformes*.

4.2.5 Ugba

Ugba (Ukpaka) is prepared by the fermentation of seeds of the African oil bean (*Pentaclathra macrophylla*). It is in great demand in the southeastern parts of Nigeria where it is consumed by natives as a condiment. It is a low-cost easily available protein source for native people (Sanni et al., 2002). Its production is also as laborious and time-consuming as dawadawa production. For its production African oil bean seeds are boiled for 12 hours until they are soft (Fig. 4.6). Seeds are then dehulled to separate cotyledons. Cotyledons obtained were sliced into thin slices and are soaked

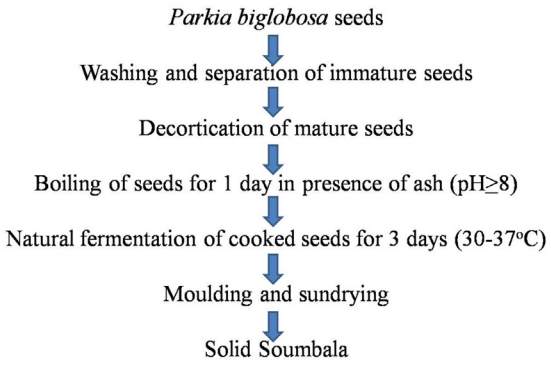

FIGURE 4.3 Different steps in production of Soumbala. *Modified from Somda et al. (2014).*

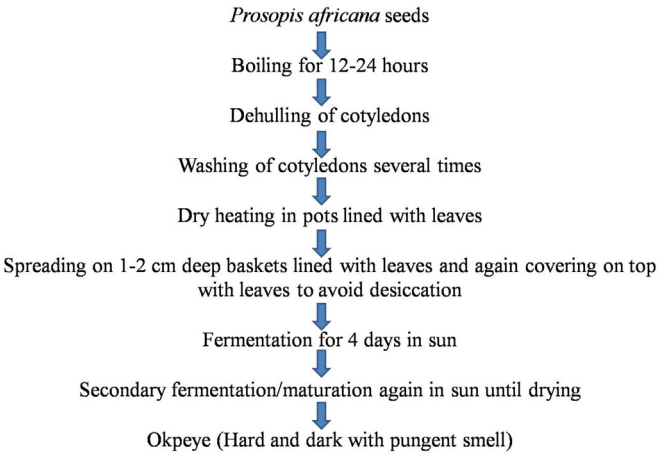

FIGURE 4.4 Traditional preparation of okpeye. *Modified from Okpara & Ugwuanyi (2017).*

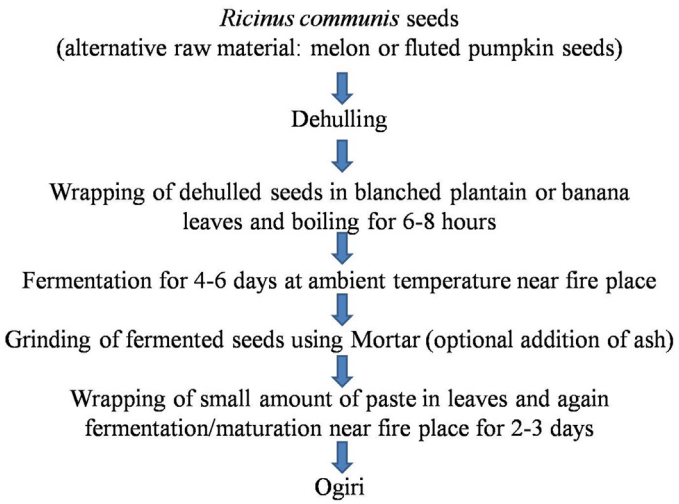

FIGURE 4.5 Traditional preparation of ogiri. Source*: Omafuvbe, B.O., Falade, O.S., Osuntogun, B.A., & Adewusi, S.R.A. (2004). Chemical and biochemical changes in African Locust Bean (*Parkia biglobosa*) and Melon (*Citrullus vulgaris*) seeds during fermentation to condiments.* Pakistan Journal of Nutrition*, 140–145. doi:10.3923/pjn.2004.140.145.*

overnight. After soaking they are washed and wrapped in banana or okra leaves and is fermented for various lengths of time. If ugba is to be consumed as a side dish or snack, it is fermented for 5–6 days; if it is to be used as a soup condiment, then it is fermented for 7–10 days (Isu & Ofuya, 2000). The main microorganism responsible for the production of Ugba is *B. subtilis* but some other species like *B. pumilus*, *B. coagulans*, *B. megaterium*, etc., have also been isolated (Isu & Ofuya, 2000).

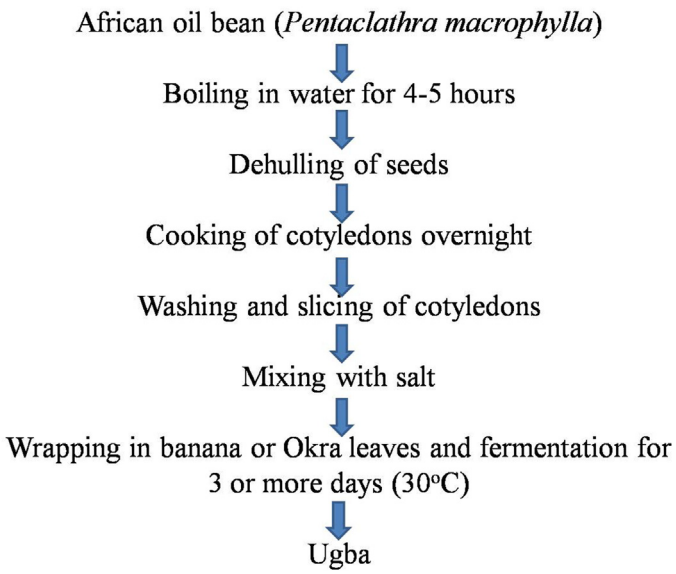

FIGURE 4.6 Flow chart for the traditional production of ugba. Source*: Odunfa and Oyeyiola (1985).*

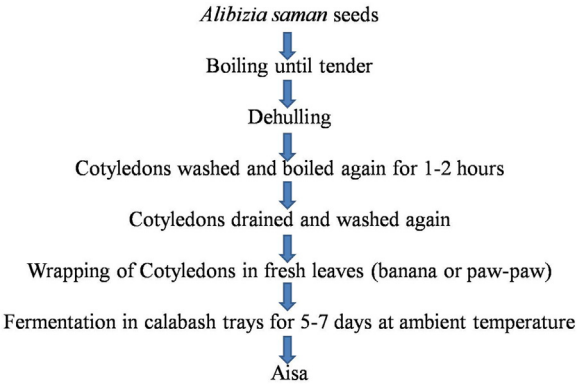

FIGURE 4.7 Steps in the production of aisa. Source*: Ogunshe, A.A.O., Ayodele, A.E., & Okonko, I.O. (2006). Microbial studies on Aisa: A potential indigenous laboratory fermented food condiment from* Albizia saman *(Jacq.) F. Mull.* Pakistan Journal of Nutrition*, 5(1), 51–58. doi:10.3923/pjn.2006.51.58.*

4.2.6 Aisa

Aisa is a condiment produced from the fermentation of seeds of *Albizia saman*. Its method of production is the same as that of dawadawa (Fig. 4.7). In its production seeds are boiled until tender. Dehulling of seeds is done manually followed by washing of cotyledons. Washed cotyledons are boiled for 1–2 hours again. Cotyledons are then wrapped with fresh banana or paw-paw leaves and arranged in calabash trays. Its fermentation is carried up to 7 days with the pH of the product reaching about 8 (Ogunshe et al., 2006). The main microorganisms responsible for its fermentation are various species of *Bacillus*; in addition to this *Escherichia coli*, *K. pneumonia*, *Enterobacter aerogenes*, *Proteus* also have been identified.

4.2.7 Owoh

It is the alkaline fermented product of cotton seeds (*Gossypium hirsutum*) with a pH above 8.8. In its preparation, cotton seeds are boiled for two hours until they are soft (Fig. 4.8). They are then soaked overnight in water followed by their dehulling. The cotyledons are then washed with water and wrapped in banana leaves. They are then again boiled for 1–2 hours. The wraps are transferred to calabash trays or earthen pots and covered with jute bags. It is then fermented for 2–3 days in some warm place preferably near the fireplace. The fermented product is molded into balls. Owoh obtained can be used at this stage or can be smoked over charcoal or sundried to increase the shelf life and desirable aroma The main bacteria responsible for fermentation are *B. subtilis*, *B. licheniformes* and *B. pumilus* (Sanni & Ogbonna, 1991).

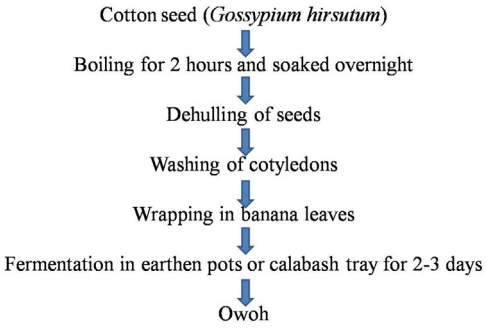

FIGURE 4.8 Traditional process of production of owho. Source: *Sanni, A.I., & Ogbonna, D.N. (1991). The production ofowoh - A Nigerian fermented seasoning agent from cotton seed (*Gossypium hirsutum *L.).* Food Microbiology, 8*(3), 223–229. doi:10.1016/0740-0020 (91)90054-6.*

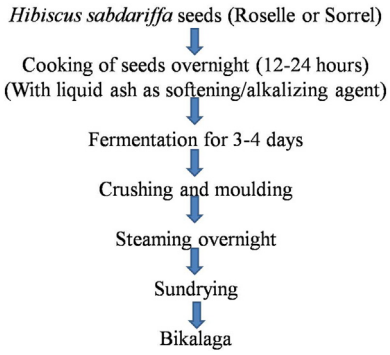

FIGURE 4.9 Flow chart for production of bikalga. Source: *Bengaly, M. & Etude, D. (2001). et valeur nutrition Int condiment traditionel riche en proteines, obtenu par fermentation naturelle des graines de* Hibiscus sabdariffa *UFR-SVT.*

4.2.8 Bikalga

Bikalga is prepared by alkaline fermentation of *Hibiscus sabdariffa* seeds and is a very popular condiment in Burkina Faso. It is known by different names in various parts of Africa. It is known as dawadawa bosto in Nigeria, mbuja in Cameroon, furundu in Sudan and datou in Mali. It is used to flavor various dishes and serve as an important source of protein, carbohydrates, lipids, amino acids, and fatty acids (Abu-Tarboush et al., 1997; Yagoub et al., 2004). In the production of bikalga, seeds are washed and cooked with liquid ash for 12–24 hours (Fig. 4.9). Liquid ash will act as a softening and alkalizing agent. Seeds are then fermented, crushed, molded, and steamed overnight. The prepared bikalga is then sundried for long time storage. The microorganisms involved in fermentation belong to different species of *Bacillus* including *B. subtilis*, *B. licheniformes*, and *B. pumilus*.

4.2.9 Soydawadawa

It is prepared from seeds of soybeans (*Glycine max*) in Nigeria. It also serves as a condiment with pH ranging between 8.2 and 8.9. In the preparation of soydawadawa, seeds are first washed and soaked overnight in water for 12 hours (Fig. 4.10). They are dehulled by hand and boiled for 2 hours. Cotyledons are then allowed to ferment for 72 hours in calabash trays stuffed with plantain leaves (Omafuvbe et al., 2000). In some places, soydawadawa is also prepared by roasting seeds rather than boiling (Dakwa et al., 2005). After fermentation, they are sundried for storage. The microorganisms isolated from soydawadawa include *B. subtilis*, *B. firmis*, *B. pumilus*, *B. licheniformes*, and *B. cereus* (Amoa-Awua et al., 2006; Terlabie et al., 2006).

4.3 Some alkaline fermented foods from Asia

4.3.1 Kinema

It is an alkaline fermented soybean food prevalent in the Eastern Himalayas. It is prepared from the seeds of the yellow cultivar of soybean (*G. max*). Seeds are first washed and soaked in water overnight (Fig. 4.11). They are then transferred to freshwater in a container and boiled for 2–3 hours. Boiled seeds are then cracked slightly with a wooden mortar and pestle to split the cotyledons. To maintain the alkaline condition, firewood ash (1%) is added to the cracked

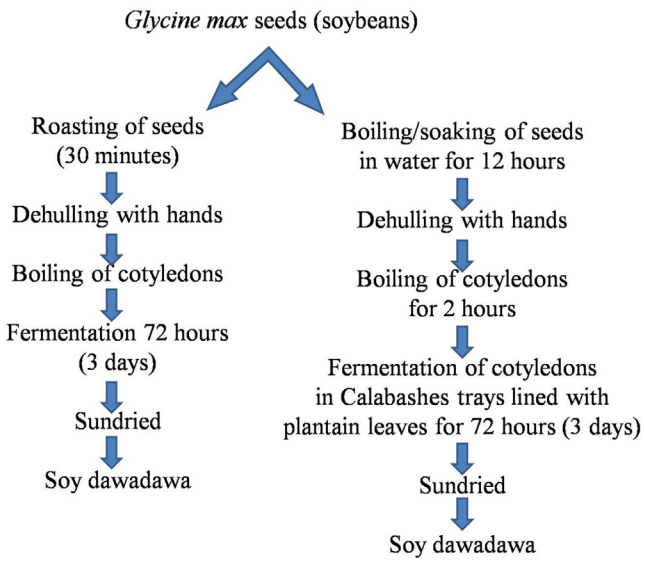

FIGURE 4.10 Two methods of production of soydawadawa: roasting and soaking (Omafuvbe et al., 2000).

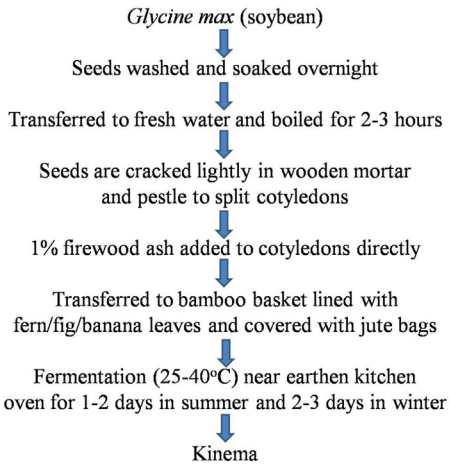

FIGURE 4.11 Traditional method of production of kinema (Sarkar et al., 1994).

cotyledons directly. It is then transferred to bamboo baskets already lined with fresh leaves of a fern (*Glaphylopteriolopsis erubescens*). In some places, people use fig (*Ficus*) leaves or banana leaves for wrapping the cotyledons. The basket is then covered with jute bags and kept for fermentation near the earthen kitchen oven for 1−2 days in summer and 2−3 days in winter. Kinema thus obtained shows the formation of white-colored viscous mass with ammonical odor. It has a shelf life of about 2−3 days in summer and about 1 week in winter. For long term storage, it is sundried for 2−3 days. The preparation of Kinema varies from one place to another and even differ among different families. Microorganisms isolated include *B. subtilis*, *B. thuringiensis*, *B. licheniformis*, *B. cereus*, and *B. sphaericus* (Sarkar et al., 1994, 2002; Tamang, 2001).

4.3.2 Hawaijar

Hawaijar is an alkaline fermented product prepared from small-seeded soybean in the Indian state of Manipur (Jeyaram et al., 2009). Its method of production is similar to kinema but in its production, the cracking of seeds and the addition of ash is not practiced (Fig. 4.12). It can be kept up to 7 days without refrigeration and its shelf life can be increased to several weeks by sun drying it for 2−3 days. Despite the popularity, it is not produced on large scale. It is still prepared at the household level by people.

In addition to this, some other fermented products of soybean are also prepared similar to kinema with a slight change in the period of soaking, cooking, and fermentation as well as the type of leaves used. Raw material and

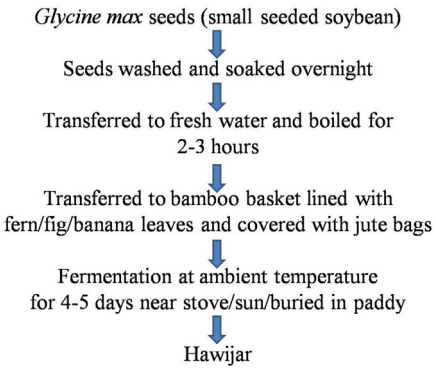

FIGURE 4.12 Steps involved in the production of hawaijar (Premarani & Chhetry, 2010; Tamang et al., 2012).

conditions required for the production of some more fermented products (tungrymbai, bekang, and peruyyan) from India are shown in Table 4.3.

4.3.3 Natto

Natto is a popular alkaline fermented product prepared in Japan. In its preparation, small soya bean seeds are first washed and soaked overnight. The soaked seeds are then cooked to soften them for 2−3 hours. The cooked seeds are then wrapped with rice straws having natural *Bacillus natto* and fermented for 20 hours (Fig. 4.13). Nowadays, the seeds are soaked and then placed in a steaming shelf and steamed at 121°C (249.8°F). They are then cooled and artificially inoculated with *B. natto* culture in a rotary cask (Fig. 4.14). Then about 50−100 g of soya bean mash is wrapped in a piece of perforated polythene film or the mash is transferred to polystyrene or wooden trays (Wilson, 1995). These trays are then stacked in the fermentation room at 30−40°C for 24 hours. The fermented product is then transferred to cold storage or is transported to the market.

4.3.4 Chungkookjang

It is a traditional fermented soybean paste used by the people of Korea. It is known to have various health benefits. It is similar to Japanese natto but has a stronger aroma. In its preparation, the soybeans are washed and soaked overnight (Fig. 4.15). The beans are then cooked for 3−4 hours until soft and fermented for 48 hours. The fermented product is then ground by mortar and pestle. Traditionally, it is fermented for 72 hours by naturally occurring bacteria in rice straw (Yang et al., 2013). The microorganisms involved is *B. subtilis*.

4.4 Fish-based alkaline fermented products

Fish-based alkaline fermented products are also used as condiments in many African countries particularly Ghana, Egypt, and Nigeria (Table 4.4). It is a very cost-effective way of preserving fish, which otherwise deteriorates very fast. Depending upon the type of product, its fermentation is carried from a few days to several months. Some of the popular condiments obtained from the fermentation of fish in Africa are lanhouin, momoni, and feseekh.

4.4.1 Lanhouin

It is prepared by the fermentation of Cassava fish (*Pseudotolithus senegalensis*) in West Africa. It is used for adding flavor to many dishes, especially soups. For its production, the fish is first scaled and gutted (Fig. 4.16). It is then treated with salt and fermented for 3−8 days (Anihouvi et al., 2005). It is mostly a spontaneous and uncontrolled fermentation The amount of salt used ranged from 20% to 35% of fish weight. To remove extra salt from fermented fish, it is washed and then dried under the sun. Traditionally, the fermentation is carried out generally in unhygienic conditions. The principal microorganisms involved in fermentation are various *Bacillus* species. In addition to this, some other bacteria like *Staphylococcus, Micrococcus, Pseudomonas, Corynebacterium, Achromobacter, Alcaligenes* have also been recovered.

TABLE 4.3 Alkaline fermented food from Asia.

Raw material	Product name	Region/ country	Microorganism	References
Glycine max	Kinema	India	*B. subtilis*, *B. sphaericus*, *B. licheniformis*, *B. thuringiensis*, *B. circulans*, *Enterococcus faecium*, *Geotrichum candidum*, *Candida parapsilosis*	Sarkar et al. (1994), Tamang (2003)
	Hawaijar	India	*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. cereus*, *Staphylococcus aureus*, *S. sciuri*, *Alkaligenes* spp., *Proteus mirabilis*	Jeyaram et al. (2008), Singh et al. (2014)
	Aakhone	India	*B. subtilis*, *Proteus mirabilis*	Singh et al. (2014)
	Bekang	India	*B. subtilis*, *B. pumilus*, *B. brevis*, *B. licheniformis*, *B. sphaericus*, *B. circulans*, *B. coagulans*, *Lysinibacillus fusiformis*	Chettri and Tamang (2015)
	Peruyaan	India	*B. subtilis*, *B. amyloliquefaciens*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Vagococcus lutrae*	Singh et al. (2014)
	Tungrymbai	India	*B. subtilis*, *B. licheniformis*, *B. pumilus*	Chettri and Tamang (2015)
	Natto	Japan	*B. subtilis* var. natto	Kiuchi (2004)
	Thua-nao	Thailand	*B. subtilis*, lactic acid bacteria (LAB)	Dakwa et al. (2005), Visessanguan et al. (2005), Inatsu et al. (2006)
	Douchi	China, Taiwan	*B. amyloliquefaciens*	Peng et al. (2003)
	Chungkookjang	Korea	*Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. thermoamylovorans*, *Lactobacillus* sp., *Pediococcus* sp., *Lactococcus* sp., *Aspergillus oryzae/A. sojae*, *Rhodotorula* sp., *Sacchromyces* sp.	Nam et al. (2012), Baek et al. (2010)
	Yandou	China	*Bacillus subtilis*	Quin et al. (2013)
	Meju	Korea	*Bacillus subtiis*, *Aspergillus oryzae*	Yang et al. (2012)
	Shoyu (Soya sauce)	Japan, Korea, China	*Aspergillus sojae*, *Aspergillus oryzae*	Nishinari et al. (2018)
	Tempe	Indonesia, Japan	*Rhizopus oligosporus*, yeast, LAB	Nurdini et al. (2015)
	Miso	Japan	*Pediococcus halophilus*, *Aspergillus oryzae*, *Zygosaccharomyces rouxii*	Nishinari et al. (2018)

4.4.2 Momoni

In Ghana the fermented fish called momoni is very popular. It is prepared from a variety of fish such as African jack mackerel (*Caranx hippos*), barracuda, catfish, and sea bream. During the processing of fish, scales and gut is removed (Fig. 4.17). The fish is then salted by adding up to 30% salt. It is then fermented for 1−5 days. Fermented fish is then cut into small pieces and sundried. Microorganisms isolated include *B. subtilis, B. licheniformes, B. megaterium, B. mycoides, Staphylococcus* spp., and LAB.

4.4.3 Feseekh

Feseekh is an important food fish or condiment prepared by fermentation of Bouri fish (*Mugil cephlus*), Pebbly fish (*Alestes baremoze*) and Tiger fish (*Hydrocyrus* sp.). Depending upon the concentration of salt used two types of feseekh

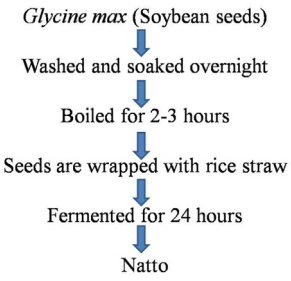

FIGURE 4.13 Traditional method of production of natto.

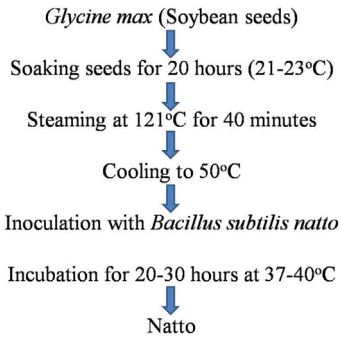

FIGURE 4.14 Modern method of production of natto. Source*: Wei, Q. & Chang, S.K.C. (2004). Characteristics of fermented natto products as affected by soybean cultivars.* Journal of Food Processing and Preservation, 28*(4), 251–273. doi:10.1111/j.1745-4549.2004.23047.x (Wei & Chang, 2004).*

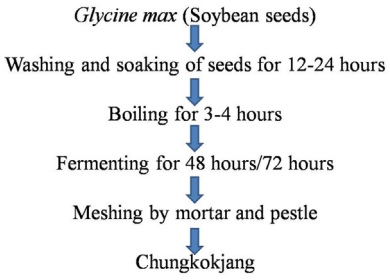

FIGURE 4.15 Different steps in the preparation of chungkookjang. Source*: Yang, H.J., Kim, H.J., Kim, M.J., Kang, S., Kim, D.S., Daily, J.W., Jeong, D.Y., Kwon, D.Y., & Park, S. (2013). Standardized chungkookjang, short-term fermented soybeans with* Bacillus lichemiformis*, improves glucose homeostasis as much as traditionally made chungkookjang in diabetic rats.* Journal of Clinical Biochemistry and Nutrition, 52*(1), 49–57. doi:10.3164/jcbn.12-54.*

are sold in Egypt. In the first type of feseekh, fermentation of fish is carried out in low salt concentration and is suitable for consumption after 15–20 days; whereas, in the second type of feseekh, salt is used in high concentration and it can be consumed even after 2–3 months of storage (Fig. 4.18). In Egypt, it is popularly consumed as an appetizer or a main dish during the feast (El-Sebaiy & Metwalli, 1989). Microorganisms responsible for its fermentation have not been identified yet.

4.5 Significance of alkaline fermented food

Food fermentation is an important enterprise useful in boosting the economy of many communities. Foods derived from the alkaline fermentation form an important component of human diets worldwide. It is used as the seasoning agent in many rural populations worldwide. Recently, due to increased awareness of people about the nutritional values and health benefits of alkaline fermented food, they are in large demand among people in villages and urban areas. They are very important in the diet of developing countries like Africa and a few Asian countries' and help in ensuring their food security among the poor population. Most of the raw materials used in the preparation of alkaline fermented food are mostly unfit for human consumption due to the presence of antinutrients and toxins. These are broken down and converted to essential amino acids thereby helping the consumer to easily digest and absorb.

4.6 Modern approach in food fermentation

In the majority of Africa, people still rely on old traditional ways of fermentation, but in urban areas people are gradually shifting to modern food processing technologies for commercial production. Recently, in countries like Asia and

TABLE 4.4 Fermented fish products.

Raw material	Product name	Region/country	Microorganism	References
Cassava fish (*Pseudotolithus senegalensis*) Spanish mackerel (*Scomberomorus tritor*)	Lanhouin	Benin/Togo/Ghana	*Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp., *Pseudomonas* sp., *Streptococcus* sp., *Alcaligenes* sp., *Achromobacter* sp., *Corynebacterium* sp.	Anihouvi et al. (2005)
African Jack mackerel (*Caranx hippos*), catfish, barracuda, sea bream	Momoni	Ghana	*Bacillus* sp., *Micrococcus* sp., *Staphylococcus aureus*, *Staphylococcus* sp., *Pediococcus* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Klebsiella* sp., *Debaryomyces* sp., *Hansenula* sp., *Aspergillus* sp.	Abbey et al. (1994)
Alestes baremoze, Hydrocynus sp.	Feseekh	Egypt	Not reported	Abd-Allah (2011)
Lampam java, Pontius gonionotus, Black tilapa (*Oreochromis mossambicus*)	Pekasam	Malaysia	Not reported	Ida Muryany et al. (2017)
Punti fish (*Puntius sophore*)	Shidal	India	*Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp.	Muzaddadi and Basu (2012)
Puntius sophore	Nagri	India	*Lactococcus lactis* subsp. cremoris, *L. plantarum*, *Bacillus subtilis*, *Candida* sp., *Saccharomycopsis* sp.	Thapa et al. (2004)
Esomus danricus	Hentak	India	*Lactococcus lactis, L. plantarum, L. fructosus, L. amylophilus, Bacillus subtilis, B. pumilus, Saccharomycopsis* sp., *Candida* sp., *Micrococcus* sp., *Enterobacter faecium*	Thapa et al. (2004), Thapa (2016)
Shellfish, shrimp	Jeotgal	Korea	*Achromobacter* sp., *Bacillus* sp., *Brevibacterium* sp. , *Sarcina* sp., *Flavobacterium* sp., *Torulopsis* sp. *Halobacterium* sp., *Leuconostoc* sp., *Micrococcus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Saccharomyces* sp.	Koo et al. (2016)
Crucian Carp, mackerel	Narezushi	Japan	*Lactobacillus plantarum, L. acidipiscis, L. alimentarius*	Tsuda et al. (2012)
Mackerel, sardine	Fish nukazuke	Japan	*Tetraaenococcus muriaticus*	An et al. (2010)
Freshwater fish (*Cyprinus carpio*)	Suan Yu	China	*Lactobacillus plantarum, Pediococcus pentosaceus, Leuconostoc* sp., *Paralimentarius* sp. *Saccharomyces cerevisiae, Hansenula anomala*	Zeng et al. (2015)
Sardines (*Engraulis japonicus*)	Bakasang	Indonesia	*Micrococcus* sp., *Streptococcus* sp., *Pediococcus* sp.	Ijong and Ohta (1995)
Raw anchovies (*Stolephorus* spp.)	Budu	Malaysia	*Micrococcus luteus, Staphylococcus ariettae*	Sim et al. (2009)

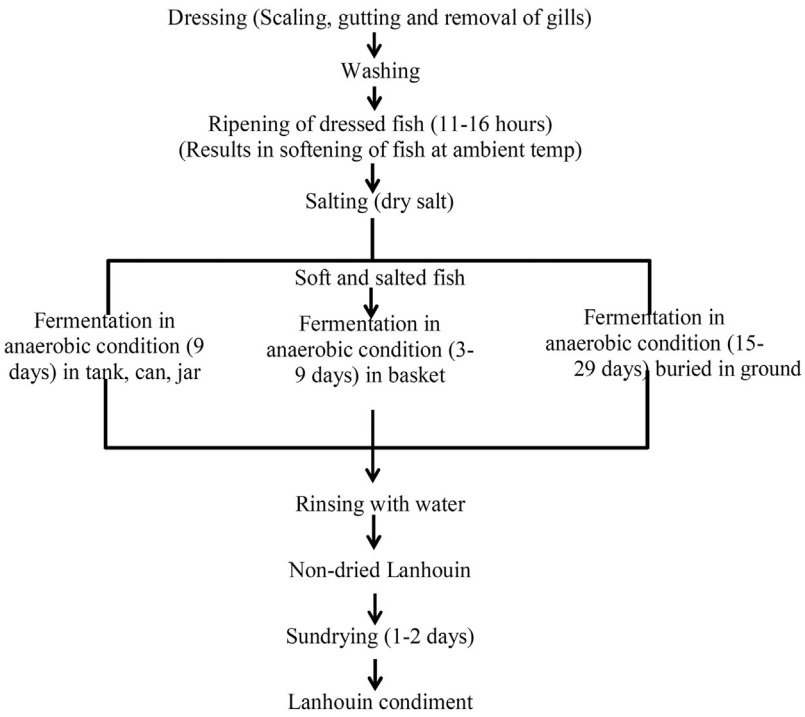

FIGURE 4.16 Flow chart of lanhouin production. Source*: Kindossi, J.M., Anihouvi, V.B., Vieira-dalodé, G., Akissoé, N.H., Jacobs, A., Dlamini, N., Pallet, D., & Hounhouigan, D.J. (2012). Production, consumption, and quality attributes of Lanhouin, a fish-based condiment from West Africa.* Food Chain*, 117–130. doi:10.3362/2046-1887.2012.009 (Kindossi et al., 2012).*

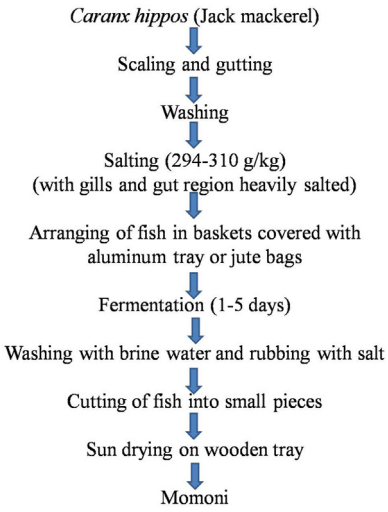

FIGURE 4.17 Production of momoni. Source*: Sheikha and Motet (2000).*

South America the old practices of alkaline fermentation are being modified (Okafor, 2007). This change from village production to new and innovative methods of production has brought out new areas of study for food scientists and small-scale industries worldwide. The alkaline fermentation food industry is not only facing the challenge of modernizing the process, but also retaining the traditional sensory peculiarity acceptable to consumer. Recently, various types of equipment have been introduced with modern technology resulting in a homogenous product of known quality, which was otherwise of uncertain quality during traditional fermentations. Although many industries have tried to make fermented products, they have ended up with a product of altered flavor. Room still exists for improvement in research, development, and flavor acceptability (Ogunshe et al., 2012). Several workers have tried to update conventional practices to curtail the manpower and time duration (Akande et al., 2010; Alabi et al., 2005).

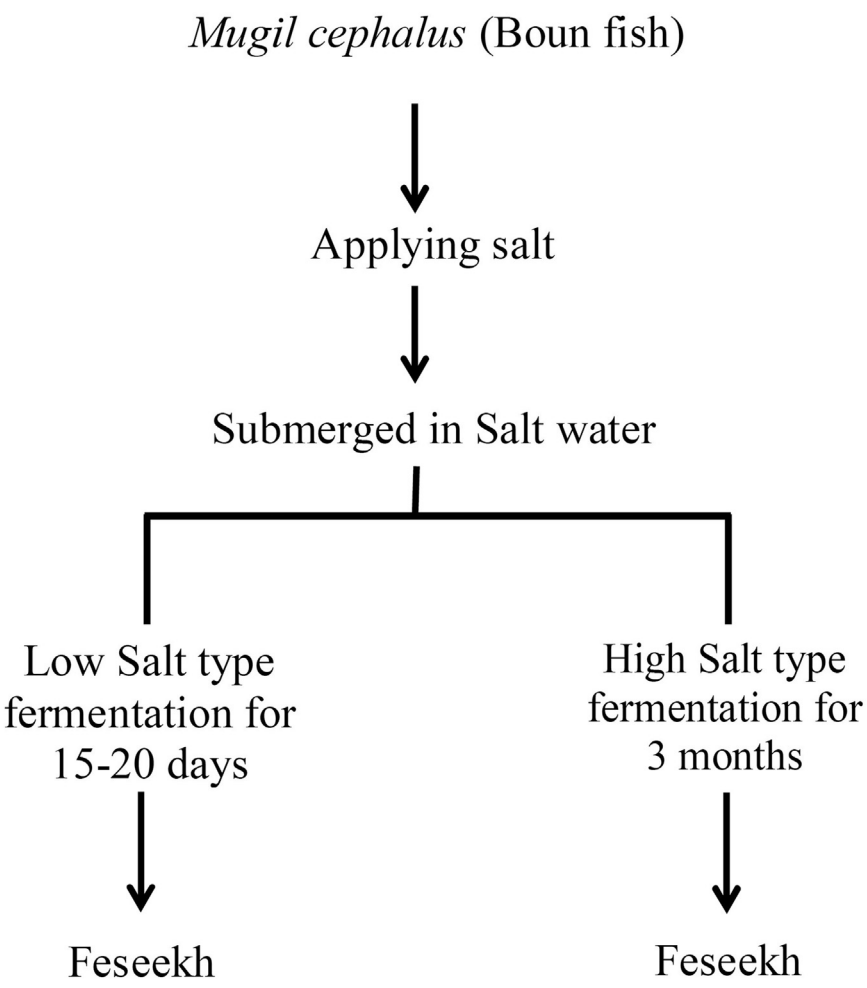

FIGURE 4.18 Method of production of feseekh (El Sheikha et al., 2014).

4.6.1 Quality and availability of raw material

Good quality raw material should be available to the people employed in fermentation technology. Farmers should be encouraged to grow improved varieties so that better and homogenous raw material is made available to the people. Most of the raw materials used for the fermentation by locals are mostly seasonal. Therefore, efforts should be made to explore alternative raw material for whole or partial replacement of seasonal raw material (Amoa-Awua et al., 2006; Okpara & Ugwuanyi, 2017). For example in the production of owoh, African yam bean or cotton seeds can be used (Ogbonna et al., 2001; Sanni & Ogbonna, 1991). Similarly, in the preparation of ogiri, the main substrate used is castor oil seed, but in the absence of these seeds, melon and fluted pumpkin seeds are used. In Africa seeds from a variety of wild trees are used in the production of alkaline fermented condiments, but due to deforestation and urbanization, the availability of raw material from forests is reduced drastically. Therefore, if we are planning to develop a small-scale industry at the village or urban level, continuous availability of raw material should be there.

4.6.2 The use of starter culture

A major challenge for the food industries involved in fermentation is the lack of standard inoculum or a starter culture. In traditional fermentation, the inoculation is fortuitous from the surrounding environment. Moreover, the process of fermentation is uncontrolled, with diverse microbial profile resulting in products with unreliable quality and stability (Ugwuanyi & Okpara, 2020). Development of competent and high-quality microbe would lead to the production of high-quality indigenous fermented condiments. The availability of effective starters will not only decrease the fermentation time but also reduce the contamination of food products by toxigenic microorganisms (Ijabadeniyi & Omoya, 2006). It has been found that the quality of the final fermented product remarkably depends upon the strain of

TABLE 4.5 ***Bacillus subtilis*** **strains and** ***Lactobacillus*** **strains screened as potential starter culture for alkaline fermentation.**

Alkaline fermented product	Potential starter culture	References
Soydawadawa	*Bacillus subtilis* $Fpdp_2$, *B. subtilis* $24BP_2$	Amoa-Awua et al. (2006), Terlabie et al. (2006)
Soumbala	*B. subtilis* B7 and B15	Ouoba et al. (2005)
Ugba	*B. subtilis* MM-4:B12	Sanni et al. (2002)
Okpehe	*B. subtilis*	Oguntoyinbo et al. (2007)
Kinema	*B. subtilis* KK-2:B_{10} *B. subtilis* GK-2: B_{10}	Sarkar and Tamang (1995), Tamang and Nikkuni (1996)
Thua-nao	*B. subtilis* TISTRO (B10TEC7123)	Visessanguan et al. (2005)
Som-fug	*Lactobacillus plantarum, Pediococcus acidilactici, P. pentosaceus*	Riebroy et al. (2008)
Mackerel mince	*L. plantarum, L. helveticus, L. lactis* subsp. *lactis*	Yin et al. (2002)
Shan yu	*L. plantarum* 120, *L. plantarum* 145, *P. pentosaceus*	Zeng et al. (2015)

microorganisms involved. Traditional culture methods are very limited in analyzing various microorganisms involved. Many studies have been going on using Polymerase chain reaction (PCR) and gene sequencing to study and screen suitable and efficient microbes for food fermentation. In addition, high-throughput sequencing (HTS) and omics technologies are very promising to perceive the efficacy of microorganisms in foods (Sirangelo, 2018). Ultrasonic waves are also being used in the food industry to develop various genuine and trustworthy food processing techniques; low intensity ultrasound has been used for improving the processes of fermentation at the desired level. It modifies the substrate as well as improves the quality of starter cultures, resulting in the production of a product with improved taste, aroma, and nutritional quality (Ojha et al., 2017). All this help in the establishment of good quality starters with the objective to achieve high-quality bio transformation. For commercialization and upgrading alkaline fermentation at the industrial level, scientists are busy in isolating and developing efficient strains of different microorganism involved in alkaline fermentation (Achi, 2005). Several strains of *B. subtilis* and *Lactobacillus* have been identified which can be used as a starter culture for alkaline fermentation of a variety of raw material (Table 4.5). For example, in Japan pure culture of *B. subtilis* var. *natto* is used in the large scale production of natto (Kiuchi, 2004; Wang & Fung,1996). *B. subtilis* kk-2:b_{10} is used for the production of kinema (Tamang, 1999) similarly, *B. subtilis* 24 BP and *B. subtilis* fpdp2 have been screened for the production of high-quality soydawadawa with good aroma (Amoa-Awua et al., 2006). For the production of som-fug, a fermented fish from Thailand, some strains of *Lactobacillus*, for example, *Lactobacillus plantarum* 145, *L. plantarum* 120, *Pediococcus pentosaceus* and *P. acidilactici* are used (Riebroy et al., 2008). Similarly for the production of suan yu, a fermented fish product from China, selected strains of *L. plantarum* 145, *L. plantarum* 120 and *P. pentosaceus* 220 are used as mixed starter cultures, resulting in improved quality and reduction of the fermentation time (Zeng et al., 2015).

An antimicrobial producing strain of *Lactobacillus* has been also screened for the production of ogi which inhibits the growth of other spoilage fungi during fermentation (Sanni et al., 1999). LAB and yeast strains have the ability to produce lysine and methionine and have been used to improve fermented foods (Teniola & Odunfa, 2001). In addition to the bacterial starter, fungal starter cultures have also been developed for alkaline fermentation. A mixture of *Aspergillus* and *Actinomucor* is used for the fermentation of the fish-product surimi (Zhao et al., 2017; Zhou et al., 2014). Giri et al. (2009) have documented the benefits and organoleptic properties of four mold starters available commercially for use. Although a number of starter cultures have been designed by many workers, still a lot of work is required in this field to improve the quality, taste, and shelf life of these traditional and seasoning agents (Table 4.4).

4.6.3 Standardization of fermentation process

The traditional fermentation is mostly uncontrolled in nature (Byakika et al., 2019). Standardization of the process is only possible if we have a sound knowledge of the various steps of the fermentation as well as other pretreatments

(Akande et al., 2010; Alabi et al., 2005; Oguntoyinbo et al., 2007; Omafuvbe et al., 2002). Equipment used in traditional processes is usually primitive and unhygienic due to various biological, chemical, and physical hazards that have been reported in many instances (Oguntoyinbo, 2014). Different agencies are working in various parts of the globe to device some new equipment and techniques to upgrade fermentation processes. There is a need to have an integrated approach of all branches of science for the standardization of fermentation processes (Mishra et al., 2017). Mechanization and modernization of fermentation and processing units can go a long way in improving the economy of the region and its people (Ugwuanyi & Okpara, 2020). In many regions, people have also started using new modified methods and modernized infrastructures. In Burkina Faso, during the production of soumbala, the use of a new dehulling machine has reduced the dehulling and boiling time of seeds by 75% (Sawadogo-Lingani et al., 2003). In Uganda, to prolong the shelf life of obushera, a fermented product, techniques like pasteurization and refrigeration have been introduced (Byaruhanga & Ndifuna, 2012). Similarly, the use of improved methods in the production of dawadawa has resulted in a reduction in the cost of energy and duration of fermentation (Alabi et al., 2005; Iwuoha & Eke, 1996). Scientists are studying various parameters involved in fermentation like quality of raw material, substrate particle size, process variables like fermentation, temperature, pH, duration, and inoculum size (Oguntoyinbo et al., 2007; Omafuvbe et al., 2002).

4.6.4 Packaging

Consumer preference is always towards attractively packed long shelf life products. Good packaging is very important for proper storage and marketing of any product (Ugwuanyi & Okpara, 2020). The longevity of products can be increased by proper hygienic packaging (Peter-Ikechukwu et al., 2016). This will reduce the chances of the postfermentation proliferation of toxin-producing microorganisms as well as undesirable changes in the aroma and flavor of the product. These fermented foods and condiments are packed by wrapping them in fresh leaves and newspapers, or kept in nonsterile open bowls or baskets which results in postproduction contamination (Okpara & Ugwuanyi, 2017). The adoption of modern, sterilized, and esthetic packaging will be an important step for the future and growth of this industry. Most indigenous fermentations are carried out without observation of good manufacturing practices (GMP) and good hygiene practice (GHP). Hazard analysis critical point (HACCP) principles should be applied during production. The occurrence of enterotoxigenic, *B. cereus* in alkaline fermented food condiments is common (Ouoba et al., 2008; Oguntoyinbo et al., 2010; Thorsen et al., 2011). Therefore, it is important to employ good manufacturing processes to ensure that any process failure does not result in health risks to consumers.

4.7 Conclusion and the future prospective

The alkaline fermented foods and condiments have long been contributing to the economy of rural indigenous people. Besides boosting the economy, it also gives a sense of food security to the poor. The cheap raw material which is otherwise inedible can be converted into a readily available source of nutrition along with delicacy. Earlier, irrespective of health benefits, the fermented foods were known and used by the members of the native community only. Moreover, they were never advertised as is done in the case of established food industries. Knowing their health benefits and rich flavor, these condiments are now promoted by people in producing countries. As a result of this and contacts of so many people in social networking sites, there is an inclination of people toward these foods, under the category of natural food. With traditional kitchen technologies and the use of conventional raw materials, this increase in demand is not matched with its production and market supply. To meet the demand for such foods, there is a need for modernizing this technology and adopting GMPs. Recent investigations in the biochemical and microbiological field have helped in improving the quality of fermented foods in many ways. In addition to this use of PCR, gene sequencing, HTS, omics technology, and use of ultrasonic waves, the future of alkaline fermentation appears to be bright and promising. A lot of exercise needs to be done by industrialists, food scientists, and researchers to generate new ideas and innovations in this area. The people engaged in this process are mostly ignorant of GMP and GHP. The government or nongovernment organization should organize training workshops to help make people aware. The use of GMP and HACCP protocols by producers will help to improve the quality of products to satisfy the needs of consumers, especially in the urban areas.

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Chapter 5

Recent trends in intellectual property rights protection in fermented dairy products

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5.1 Introduction

Fermentation technology is used for the production of various food products with enhanced properties to increase nutritional value, digestibility, better aroma, increase in shelf life, presence of probiotic microbes, microbial stability and better storage condition. The fermented foods are used since long ago by African and Asian countries due to its rich in nutritional value along with functional properties. Traditionally, ethnic population uses locally available sources of cereals, grains, milk and vegetables for the preparation of fermented food based on their environmental condition. The methods for making traditional fermented foods followed one generation to the next, such as buttermilk or yogurt, famous globally in the food market; and the knowledge of making traditional fermented food is adopted and modified by the food industries. Current food is undergoing many industrial processing starts from pasteurization affecting nutrients of the food such as amino acids, fibers, minerals. Fermentation is not only having health benefits but also relating to safety and food preservation. Hence, in developing countries fermented food can be protected in rural area by generating income at small scale farms.

For the industrial sustainable fermented food production, sound knowledge of the microbial world and enzymes at molecular level is required. Fermentation is the best way to preserve highly perishable food sources. Fermentation can be classified into two types: aerobic and anaerobic. During the process of fermentation, microorganisms break down the fermentable sugar into alcohol, organic acids and carbon dioxide along with the release of anti-bacterial metabolites that inhibit the growth of food pathogen from food (Tamang et al., 2016).

Globally, ethnicities or religions and environmental conditions have influenced dietary habits. Different cultures, regions and communities have varieties of dietary habits depending upon accessibility of plant and animal resources. In the different parts of the world based on the predominant type of cereals-grain productions, food habits can be classify into three types: (1) porridges from maize preparation in South America and African countries, (2) main staple food is rice in Asian countries, and (3) bread made from wheat and barley in Europe, Australia, North America and western Asia region. Whereas, dairy products and milk is consumed all over the world. For past years, alcoholic beverages and fermented food were produced in absence of a starter culture by traditional fermentation methods using animal and plant sources. In the 20th century, by vigorous research and study, the starter culture was made available for industrial fermented food production. Because of microbial contamination, food spoilages is a normal process, but scientists have repeatedly found positive quality results under controlled conditions such as different taste and long-lasting food products. Even in the absence of microbiology, people made fermented food at home. In the traditional home fermentation method a small portion of previously fermented food is added into fresh substrate to initiate the process (Makino et al., 2010). The presence of microorganisms depends on climatic condition, raw material and methods of preparation. The multiplication of microbes may include spoilage microorganism present in the food, but the initial stage of fermentation secretes organic acids and, by reducing pH, suppresses the spoilage microorganisms (Bourdichon et al., 2012).

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5.2 Nutritional benefits

5.2.1 Probiotics

Functional microorganisms that exist in fermented foods contain antioxidant and probiotic properties that have shown various health benefits to consumers. Functional microorganisms enhance the chemical components present in animal or plant sources with bio-preservative effects, fortifying with bioactive components, enriching with sensory properties, reducing toxic components, releasing antimicrobial and antioxidant compounds. Globally, fermented foods prevent cardiovascular disease, diabetes, gastrointestinal problems, certain cancers, lactose intolerance, urinary tract infection, antibiotic associated or infant diarrhea and irritable bowel syndrome. Live microbes including yeast and bacteria known as probiotics have beneficial effects on human health (Hinrichs & Stoeckel, 2017). Probiotic microorganisms are added into various types of fermented food for the past few years. The various microorganism such as *Bacillus* species, *Lactobacillus*, *Leuconostoc, Bifidobacterium* and *Propionibacterium* isolated from cheese, species of *Rhizopus, Amylomyces, Penicillium, Aspergillus, and Neurospora, Saccharomycopsis, Candida, Torulopsis, Geotrichum, Debaryomyces, Schizosaccharomyces, Torulopsis, Mucor, Actinomucor* and hundreds of others are found in many fermented foods (Ravinder et al., 2012). The properties of microorganisms to be used in the production of functional foods includes antioxidants, therapeutic, poly-glutamic acid, degradation of toxic components, fibrinolytic activity, peptide production and nutraceuticals properties.

When the live microbes are added to fermented foods, there are many factors that affect the survival of probiotics in the food products while entering the gastrointestinal tract of the individual. The factors include product storage condition, interaction of starter culture with live microorganisms, the chemical composition of the product and the growth phase of probiotics.

5.2.2 Fermentation and nutritional quality of food

Fermented foods are highly nutritious as compared to non-fermented food in many ways. Firstly, during fermentation microbes not only participate in catabolic reaction, but also are anabolic and synthesize many growth factors and vitamins. Secondly, cellulose, polymer and hemicellulose from plant materials are not digested by humans, but through enzymatic digestion; complex carbohydrates change into digestible components. Enzymes include phytase, lipases, amylases hydrolyze phytates, lipids and polysaccharides, thus inreasing the level of iron, protein by decreasing the antinutrients concentration such as tannins. Hence, the fermentation process enhances the nutritional value of food such as fruit juice (Bourdichon et al., 2012). Third, in the case of seeds and grains, nutrients are trap inside the cell wall as indigestible material even after cooking, but by using cell disruption techniques they change into digestible nutrients, such as protein. The food fermentation process with the help of microbes breaks down the cell wall and makes available all the nutrition for human digestion.

5.2.3 Intestinal pH balance

Indigenous microbes present in human body work collectively toward improving intestinal health. The bacteria play an important role in preventing colonization of pathogens in a healthy gut. The large intestine has acidic pH which destroys the putrefactive bacteria responsible for damaging the gut and producing foul smells. Fermented food includes *Bifidobateria* and *Lactobacillus* strain−producing acids which keep the intestinal pH healthy. At an acidic pH, healthy positive bacteria inhibit the growth of pathogenic bacteria and mold. Along with the indigenous bacteria, there are many disease-causing bacteria including protozoa and fungi residing in the body at low concentrations. Bad microbes can release toxins in food which may affect human health and are responsible for many diseases (Oliveira et al., 2001). But in a healthy normal person, good bacteria residing in colon prevents colonization of pathogenic microbes in the gut.

5.2.4 Alleviation of lactose intolerance

When people lack β-galactosidases activity, also called lactase, in small intestine, they are unable to digest lactose milk sugar that results in symptoms of diarrhea, abdominal pain, nausea and bloating. The condition is known as lactose intolerance, which is most common in non-white adults. In healthy adults, lactose can easily hydrolyze by the enzyme lactase into glucose and galactose, finally cleared by the liver. Whereas, in lactose intolerant adult's, lactose does not hydrolyze and pass to the colon where it ferments and releases osmotic active products causing symptoms of intolerance

(Wilt et al., 2010). Fermented dairy products are found to be useful in the digestion of lactose because it contains *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strains of microorganisms. It has been found that individual suffering from lactose intolerance can easily digest fermented dairy products.

5.2.5 Biodegradation of phytase

The food containing phytic acid includes seeds, legumes, nuts and grains containing phosphorous, which plays important role in the formation of cell membranes and energy production. But phytic acid is responsible for binding with many micro- and macronutrients, important for various metabolic processes, by inhibiting their absorption in the digestive system. Fermented food containing phytases enzymes in microbes include yeast, gram-positive bacteria, gram-negative bacteria, which can biodegrade the phytic acid. Phytases are used as food supplement or pre-treatment of food to hydrolyze the phytate. There are many grains like wheat which contain higher level of phytases, and after the fermentation of grains shows reduction in 60%−92% of phytic acid (Lopez et al., 2000).

5.3 Fermentation: cultural importance and food security

The food security is determined by (1) easy access to get enough resources for essential nutrients containing food in the regular diet; (2) accessibility of good quality and sufficient quantity of food; (3) adequate amount of food should be available all time even during climatic or economic crisis; and (4) to maintain a healthier lifestyle; proper use of clean water, food, sanitation and healthcare should be available. Fermentation processes provide the food security to the world specially to vulnerable people. Food fermentation enhances flavor, preserves perishable raw material for longer times, contains many vitamins and enzymes, increases absorption of nutrients, increases the shelf life of the products, removes anti-nutrients and is inexpensive compared to other nutrient rich food. Adding nutrients in the regular diet is very expensive, but not with fermented food. It can be prepared at home with very minimal cost and cuts down on the additional supplements needed to maintain a healthy life. Traditional fermentation used in rural areas is a substitute for the refrigerator. Fermented foods release nutrients and make them bioavailable for absorption; it helps to keep healthier life for those suffering from asthma, constipation, allergies or lactose intolerance. Under humid environmental conditions, highly perishable fruits and vegetables deteriorate very fast. Using a freezing method for long periods of food preservation is not economical and canning at small scale can be serious for human health.

5.4 Common indigenous fermented dairy products

Milk is the key constituent in all types of dairy products and the delivery agent for enriching fermented dairy products with probiotic strains. Milk is the combination of bioactive components such as minerals, proteins, lipids and sugar required to balance the gastrointestinal tract. Fermentation preserves various nutrients of milk. The research has proven that by adding different microbial cultures in milk during fermentation, it can make varieties of milk products with different flavor, taste and health benefits (Pedone et al., 1999). Globally, with the increasing demand of healthy food, fermented milk products become more popular. Fermented milk products include yogurt, lassi, buttermilk, cheese, butter, kefir, shrikhand, ice-cream, kumis and leben. Fermentation is based on environmental conditions that includes temperature, pre-treatment of milk, the fermentation method; local bacterial culture fermented milk products differ from one place to other. It is proven by various scientific studies that diseases like hypertension, certain cancers, osteoporosis, coronary heart disease and gastrointestinal disorder can be prevented by using probiotic-enriched fermented milk. Fermented milk contains a starter culture which has health benefits to keep the balance in intestinal microflora and is responsible to increase shelf life of milk by developing organoleptic characteristics. Hence, fermented dairy products are also considered as one of the important functional food. Milk contains good bacteria includes *Bifidobacteria*, *Lactobacillus acidophilus, Lactobacillus reuteri*, and *Lactobacillus casei* (Stanton et al., 2001).

Initially, fermented milk products are prepared by a wild strain of starter culture known as NFM. Depending upon the dominant strain, final product taste and quality is not predictable. Therefore, in the dairy industry fermented milk products are produced by using commercial starter cultures under controlled condition. In developing countries, rural communities prepare NFM products at house scale and have a significant source of generating income. Many wide ranges of naturally fermented products are available all over the world with different nutritional composition, depending upon their climatic condition. Among them, there are five basic similarities in all varieties of natural fermented food. First, natural fermentation is initiated by rural people under poor living conditions with limited facilities. Second, the fermentation process originates from raw milk, available container, equipment and climatic condition of the region.

TABLE 5.1 Fermented products and their potential health benefits.

Fermented milk products	Source of milk	Microbial strain	Health benefits
Cheese	Sheep milk, Buffalo's or cow milk	*Penicillium roqueforti, Lactococcus lactis subsp., L. delbueckii*	Good source of probiotics, maintenance of bone health having antimicrobial and antithrombotic properties (Kato et al., 2002)
Yogurt	Buffalo's or cow milk	*Lactobacillus bulgaricus, L. delbacillus, Streptococcus thermophilus*	Lower risk of T2D, promoted intestinal health (Chen et al., 2014)
Shrikhand	Cow or buffalo's milk	*L. bulgaricus, S. thermophilus*	Used in the treatment of acidity and gastro enteritis, diarrhea (Patel & Schauen, 1997)
Lassi	Cow or buffalo's milk	*L. bulgaricus*	Nutritional and therapeutic, Used for the treatment of diarrhea, piles, chronic specific and non-specific colitis, jaundice (Anon, 2003)
Curd	Buffalo's or cow milk	*L. plantarum, Streptococcus lactis, L lactis, L. delbrueckii*	Promotes intestinal health (Parvez et al., 2006)
Kefir	Cow, Sheep and goat milk	Lactic acid bacteria, *Saccharomyces* kefir	Good source of probiotics, anti-diabetic activity, anti-inflammatory, antimicrobial and antioxidant activities. (Rosa et al., 2016)
Cultured Butter milk	Cow or buffalo's milk	*S. cremoris, S. lactis subsp. diacetylactis*	Blood pressure and cholesterol reduction, antiviral effects, prevents from undesired stomach acid (Shiby & Mishra, 2013)
Leban	Sheep and goat milk	*L. bulgaricus, S. lactis S. thermophiles*, Lactose fermenting yeast	Reduces the risk of blood pressure, boost immune system, good for bones and digestion (Ahmed et al., 2016)

Third, locally available vessels are used for the production of fermented food, not the modified containers which are used in urban areas. Fourth, the selection of a starter culture decides the quality and shelf life of the products which is not easily replicated by industry. Fifth, the natural fermentation process incubation time of the product is 2−3 days at climatic conditions of the region without any manipulation (Digo et al., 2017). Table 5.1 shows indigenous dairy fermented products with their health benefits.

Globally, from the human health and quality point-of-view, dairy product safety is a very important issue. Milk safety structure is categorizing into two types: traditional and science based protocol. The food contamination refers any chemical agent, biological or physical, that enter into the food from a farm environment. The pathogens including *Staphylococcus aureus, Salmonella, Mycobacterium avium* and other pathogenic strains that can easily enter through unhealthy protocols while handling from contaminated surface, packaging materials, utensils, starter cultures, primary production, formulations, transportation, labeling and serving. The complete pasteurization is important to remove pathogens from raw milk; incomplete pasteurization due to insufficient temperature/time exposure results in the growth of pathogenic microorganisms responsible for reduction in shelf life and safety of the dairy products. Normally, there is presence of natural antimicrobial activities including lactoferrin and lysozyme in milk, and an antimicrobial system such as air cooling, boiling, fermentation, which protects the milk from pathogenic microorganisms. The safety hazard of dairy products is also related with raw milk processing and production by animal husbandry practice. The chances of contamination of raw milk is expected two ways. First, endogenous contamination takes place if pathogens directly transfer from infected animal blood, and, second, by exogenous contamination during collection of milk into containers near animal feces or other environmental routes (Adesulu & Awojobi, 2014). The risk of contamination also increases if farm animals or rural small dairy animals roam around in search of water and feed in grass fields and ingest pathogens or bacteria including *Bacillus*, *Streptococcus* and *Corynebacterium* which may colonize around mammary glands. Hence, it is important to assess or monitor them while grazing for feed and water on grass land (Capozzi et al., 2017).

Dairy farm workers need to be upgraded with current information about dairy product safety, good hygiene practices and hazard identification by attending training programs conducted by government authorities. Government regulatory

authorities needs to set proper safety standard protocols and conduct inspections to maintain quality and safety assessments even for small dairy farms.

Before proceeding towards the market, dairy products need to strictly undergo standard quality checks. To execute the standards, it is important to keep the various checkpoints to maintain the quality starting from the milk collection center followed by transportation till it reaches factories for processing. In dairy industries, before pasteurization the milk quality is determined by the level of microbial load and somatic cell count. If the level of microorganism and somatic cell count is high it indicates poor milk quality and is responsible for a reduction in milk protein and poor curd texture. National authorities made it mandatory to follow regular quality control standards through various quality control programs. Hence, the science-based quality control protocol, hazard analysis critical control points (HACCP) is used all over the world to maintain the quality and safety of food.

5.5 Intellectual property and technology management in dairy sector

Intellectual property (IP) relates with the original ideas created as a result of human intellect in the areas of art, literary, technical or scientific discipline. Every inventor/creator is provided with legal rights so that they can protect their invention for a specific duration of time (Singh & Singh, 2015). Intellectual property rights (IPR) helps inventors in getting exclusive rights to avail maximum commercial benefits from their property. IP plays a very important role in today's economy. IPR is a strong device which provides protection of time, money, efforts and investment by the inventor and would also ensure recovery of the research and development costs for further possible investments in research. Patents, trademarks, trade secrets and copyrights are the various types of IP protection available. If the invention is satisfying the criteria of non-obviousness, novelty and industrial application, a patent can be awarded. In the present context, IP protection is mandatory in order to avoid exploitation of such inventions for commercial profits by individuals/ organizations in India and abroad. Also, IP protection motivates the scientists/ researchers for further research and technology development as the inventor is recognized and rewarded.

Earlier IP used to include patents, industrial designs and trademarks only, but presently it has a wider coverage. IPR now supports handling infringement, unauthorized usage and piracy, as well helping in providing all the required knowledge to the public, as now all the IPs are available in the public domain except trade secrets (DST, 2002; Singh & Singh, 2015). IP protection is available for patents, industrial designs, trademarks and copyrights. A patent is a vital tool of IP governed by Indian Patent Act 1970, which is granted to a patentee to gain benefit at the commercial level. A patent can be granted for a novel product or process development. The patent is awarded for 20 years, and no patents are granted for food items and drugs. Industrial design includes modification of shape, patterns, line composition and colors. Trademarks are IP tools which include name, logo, and mark for the product/service through which the service provider can be recognized. These can be licensed, sold and bought. A copyright is related to ideas in material forms, such as musical, artistic, cinema, audio tapes, dramatic and software used in computer. All these IP efforts are opening the way for research in dairy sciences for the advancement of dairy researchers, entrepreneurs, industry and the society.

5.5.1 IP scenario of ICAR in dairy sector

In India, dairying was earlier regarded as subsidiary occupation but has now acquired an independent status. Dairying is supporting the rural families with income opportunities and providing employment. But dairy farming is still facing obstacles as updated scientific techniques and information generated by researchers have not reached the dairy farmers. As per National Agricultural Innovation Project (NAIP) (2014), it is the much-needed missing link which results in low milk production and no improvement in the milk quality. India's premier institute Indian Council of Agricultural Research ICAR set up an IP management system in 2006; information reaches the dairy farmers so that the gap can be bridged.

One of the leading institutes under ICAR is the National Dairy Research Institute (NDRI), Karnal, working in the various aspects of dairy research. Various patents are filed every year which are categorized under different subject areas of dairy research including milk processes and products, dairy machines, technologies for milk adulteration detection and animal breeding to name a few (Singh & Singh, 2015). Fig. 5.1 represents the various categories of patents filed from year 1960−2014 in dairy sciences by NDRI. Transfer of intellectual assets by commercialization of technology is an important part of protection of IP. Commercialization helps in analyzing the business and checking the technical feasibility of innovations to entering the market. The transfer of intellectual assets to various public and private organization in dairy science from NDRI are categorized in six subject areas. The various subject areas include technologies for milk adulteration detection, dairy food processing, dairy farming technologies, animal nutrition, animal food products respectively, and dairy food processing machinary (Singh & Singh, 2015).

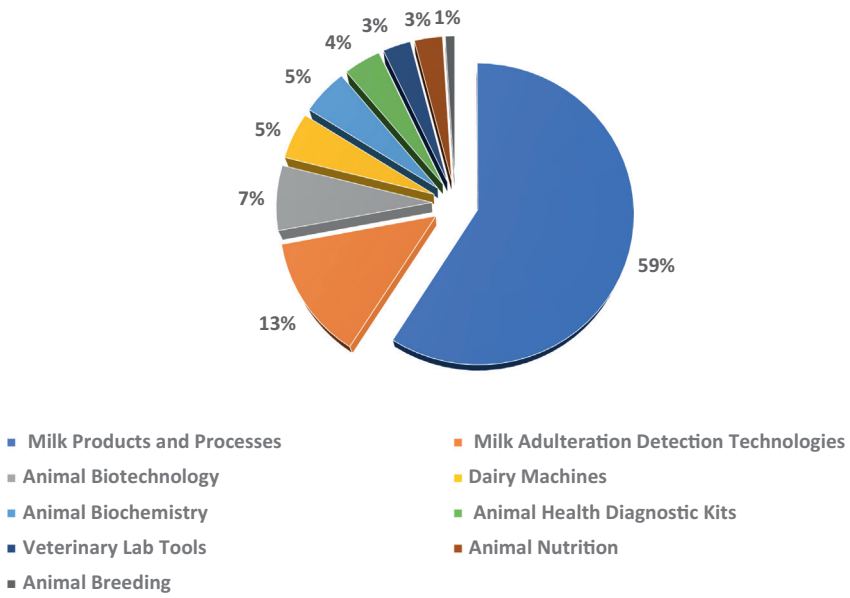

FIGURE 5.1 Figure representing various categories of patents filed from year 1960–2014 in dairy sciences by NDRI. *NDRI*, National Dairy Research Institute.

In 1997, the first technology transfer in dairy sciences was the "Khoa making machine" which is followed by another technology transfer for "Long shelf life paneer" in the year 2005. Subsequently, in 2006 five more technology transfers were reported, including process for herbal ghee preparation, milk fortified with calcium, complete automated system for Chhana balls preparation by continuous production, and machine for preparation of continuous paneer/chhana. Thirty-eight partnerships were developed from 2007 to 2015. Various technologies were transferred in the area of dairy sciences including ready to cook milk chips, dairy cattle feed containing rice bran lecithin and phospholipids, herbal ghee, acido-whey, milk fortification with calcium, biscuits fortified with iron, preparation of low cholesterol ghee by a laboratory scale process, and paneer with a long shelf life (Singh & Singh, 2015). New technologies evolved in food processing in the dairy sector included mechanized production of chhana balls; machines making continuous paneer/chhana, kits for detection of adulteration in milk, kits for β-lactum antibiotic group in milk utilizing bacterial spore in the form of biosensor and detergent in milk; micro-techniques and novel selective mediums for detection of *Enterococci* in milk; differentiating A1, A2 milk by PCR based methods; analyzing the quality of anionic detergent in milk; methods for rapid analysis of detergent in milk based on color test; analysis of urea, glucose, hydrogen peroxide and maltodextrin present in milk by strip test; and two stage enzymatic assay for analyzing the presence of *L. monocytogenes* in milk by a two-stage assay. The assets developed through continuous research were then transferred through signing contract research or service agreement to twenty-six different organization in India and abroad.

Thus, IP and its management in the dairy sector is of high importance. Protection and rights to the inventors motivates further research in the area and its security assurance. NDRI is providing encouraging results in the field of dairy sciences by filing the patents and working towards their commercialization that also motivates the scientific community. Technology partnership further involves various stakeholders including public and private organizations as well as dairy industry, so as to reach the target market and provide the latest technological innovations to the dairy farmers at the urban and rural levels.

5.6 Patents on advances in fermented dairy products

Milk and milk-based products are consumed across the globe throughout the year in huge quantity; thus, continuous research is needed for new innovations and product development and modifications. Dairy processing methodologies and products need continuous investigation so that stable, improved products can be delivered with reduced energy needs for processing. Due to the current market scenario and huge consumer demand for dairy products of high quality, with special, flavorful milks and food products having better nutritional benefits for better health, there is continuous modifications taking place based on research and technological developments in this area. Chemical modification/immobilization of enzymes may result in enhanced characteristics and better usability in the dairy and food industry. *Pseudomonas mendocina* M-37 lipase immobilized on microcrystalline cellulose showed six to sevenfold enhanced

synthetic activity, making it a suitable candidate for its application in food industry (Dahiya et al., 2010, 2014). Based on the need, special milks are now added to the milk products which includes "lactose free milk," needed for lactose intolerant population, and milk fortified with vitamins, calcium, and proteins as compared to traditionally available milk such as pasteurized, fresh pasteurized, microfiltered and long-life milk.

Since inception, there are various technical challenges faced by dairy industry requiring continuous research and development which includes: (1) developing new techniques for complete sterilization of milk without effecting its taste, color and nutritive properties; (2) improvement in delivering technique of dairy starter to the production line avoiding the undesired microorganisms; (3) reducing the time required for cheese ripening and other dairy products; and (4) improvement in physical properties of milk-related products such as stability of product, particle size, viscosity and rheological characteristics and to develop dairy products with low lipid level. In recent years, various patents filled in the area of dairy products and latest technologies were developed for the modification of products as mentioned below.

5.6.1 Patents on thermal treatment of milk

Thermal treatment of milk is done to enhance its stability, but during storage and further processing it may lead to changes in color and gelation which is undesirable. This condition is also known as browning. In the 19th century, a new technique of pasteurization was utilized for partial sterilization which can remove harmful microbes from the milk, but it was also found to change the characteristics of milk. In this context, a patent (United States 4591463A) is filed on methods and apparatus for treating liquid materials (Nahra & Woods, 1986) providing an improved apparatus and methodology for sterilization of liquid products while maintaining its physical properties and taste. It includes heating fluid (milk) at a specific temperature with minimum agitation (minimum physical stress) thus, resulting in low denaturation of whey proteins when compared to pasteurization. Production of highly concentrated milk is beneficial as it leads to decreased shipping cost and storage. But concentration of milk is difficult as it generates the problem of browning and protein gelation due to thermal processing. United States patent 8236362 B2 (Cale et al., 2012) related to a heat stable milk product, which is concentrated and added with enhancer and stabilized that is modified to be more stable and sensory pleasing. It includes concentration using ultrafiltration, the addition of a stabilizer and an enhancer which produces a stable concentrated liquid for at least 6-months' time under ambient conditions.

Patent EP 0620977 (Muzzarelli & Manzolli, 1994) is solving the issue of cooking huge amounts of curd in its whey by performing heat treatment of a liquid product using a special apparatus. It includes heat treatment of milk to produce Grana Padano/Parmigiano Reggiano, within the cooking time. Thus, this innovation provides a novel process which allows the easy handling of huge amounts of liquid for treatment and easily manages the treatment time. The process and apparatus are mainly used for preparation of heated-curd cheese, but can also be used for other types of cheese and liquid food products. Sterilization of milk by ultra-high temperature (UHT) treatment results in gelation also known as age thickening. An invention under Patent EP 1946644 A2 (Martinus et al., 2008) relates to the use of emulsifiers in small quantity to reduce the gelation in milk treated by UHT. The selected emulsifiers include monoglycerides, diglycerides, lecithins and their combinations. The patent EP 2048962 B1 (Hans et al., 2018) is based on methods for a modified technique for heat treatment of milk products. It consists of food products which are based on milk and also contain starch and other carbohydrates. The patent is mainly focusing on the process for treatment of flavored milk, fresh milk, skim milk, milk with reduced lactose content, fresh cream and various other products of milk in order to enhance their shelf life. It includes sterilization and concentration of milk, which is done by heat treatment of the product twice at high temperatures and by cold reverse osmosis process.

5.6.2 Patents on dairy starter culture

In the dairy sector, starter bacterial cultures, mainly lactic acid bacteria (LAB), are utilized for fermented cheese preparation. Such cultures are regarded as dairy starter cultures which are harmless bacteria imparting special characteristics to dairy products. The starter culture leads to lactic acid development thus eliminating the contaminating microbes. Proteolytic enzymes are produced by LAB, which contribute to the ripening of cheese by degradation of cheese proteins. The starter culture also ferments citric acid and lactose to aromatic compounds thus giving fermented dairy products specific aroma and taste. Various patents and new innovations are reported in the field of starter culture development and proteolytic enzymes for improving the yield of cheese, production of enzyme modified cheese (EMC), accelerate cheese ripening process and bioactive peptides production (Feijoo-Siota et al., 2014). Table 5.2 represents various patents filed on fermented dairy products.

TABLE 5.2 Table representing various patents filed on fermented dairy products.

Title of patent	Inventors	Novelty of patent	Patent references
Starter culture compositions	Van Pim Hee	It includes novel process for making a starter culture composition consisting of microorganism, cryoprotectant and one stimulating additive	WO2012076665A1 2012-06-14 (Hee, 2012)
Production of cottage cheese by using *Streptococcus thermophilus*	Morten Carlson, Thomas Janzen	It provides a method for increasing the yield of cottage cheese, esp. cottage cheese made using a *Streptococcus thermophilus* strain	United States 9028896B2 2015-05-12 (Carlsons and Janzen, 2015)
Heated buttermilk and cream for manufacturing cream cheese product	Scot Alan Irvin, Chad David Galer, Omar Atia	The present disclosure involves the heating of buttermilk and cream for extended duration to provide a novel flavorant. The flavorant may be used to provide low-fat dairy products, such as low-fat cream cheese, with organoleptic properties	United States 8722130B2 2014-05-13 (Irvin et al., 2014)
Process for preparing a functional dairy dessert	Péter Horváth	The object of the invention is a process for preparing functional dairy dessert characterized in that cooked cereal grist prepared with fermented milk is added to fresh fermented cheese	United States 20100119649A1 2010-05-13 (Horváth, 2010)
High protein yogurts	Matthew Galen Bunce, Rajiv Indravadan Dave	Yogurts contain fermented lactose-reduced skim milk such that the protein content of the yogurt is from 5% to 14% by weight of the yogurt. The total solids content is less than 38% by weight of the yogurt	WO2014095543A1 2014-06-26 (Bunce and Dave, 2014)
Probiotic composition (*Lactobacillus casei* strain ATCC PTA-3945)	A. Satyanarayan Naidu	The Probiotic composition comprises of strain KE01 of *Lactobacillus casei* having ATCC accession number PTA-3945	United States 6797266B2 2004-09-28 (Naidu, 2004)
Enzyme preparation for accelerating the aging process of cheese	Jeffrey T. Barach, Larry L. Talbott	The present invention is a composition for accelerating the aging process of cheese which includes a partially disrupted preparation of the lactic acid bateria *Lactobacillus casei*, *L. lactis* or a mixture and disrupted cells of *Lacto1bacillus plantarum* and dried pre-gastric lipases	EP 0150743B1 1988-05-11 (Barach and Talbott, 1988)
Reduced fat cheese having enhanced organoleptic properties	Jon Reeve, Jenni Justiz	It includes reduced fat cheese and full fat cheese which are mixed together at a ratio of 40%–50% reduced fat cheese to about 50%–60% full fat cheese	United States 20080124428A1 2008-05-29 (Reeve and Justiz, 2008)
Extended shelf life milk and process for its production	Jörg Hinrichs, Marina Stoeckel	It relates to stable milk having a stability at room temperature of at least five and preferably 6–12 months, obtained by treating the raw milk to be treated with multiple heating plates	EP 3192374A1 2017-07-19 (Hinrichs and Stoeckel, 2017)
Fermented milk nutraceuticals	Izvekova Tamara Georgievna, Kornilov Alexandr Viktorovich, Amirian Irina Surenovna	Novel cultures of *Lactobacillus acidophilus*-combination of group Rr-2 strain and *L. acidophilus* N.V. Er 317/402 used for fermented milk nutraceuticals	United States 6358521B1 2002-04-19 (Izvekova et al., 2002)

Few patents are based on mechanical device innovation to transfer the starter bacterial culture to the process line. European patent 1009243B1 (Hoier et al., 1998) discloses a device used for transfer of a liquid starter culture to the fermentation vessel. It includes a mechanical device that is adapted to help in opening a conventional container for bacterial starter strain with further transfer of the bacterial starter to the process line from the opened container maintaining

aseptic conditions. The patent WO2000013519 (Rodney, 2000) reports the use of exogenous protease along with the starter *Lactococcus lactis* to avoid undesirable flavor generation in the ripening of cheddar cheese on the basis of its intracellular aminopeptidase activity (PepN and PepXP). Another patent, EP 0810289 (Germond et al., 2004), discloses production of recombinant strains of *Lactococcus lactis* MG1363 resulted in faster proteolysis and thus the ripening process. Similarly, accelerated maturation of cheese (cheddar and Gouda) is reported by another patent, United States 20070160711 (Dijk et al., 2007). It discloses the addition of carboxypeptidase with the coagulant. Carboxypeptidases CPD I is characterized as (Degan et al., 1992) from *Aspergillus niger* N400. Carboxypeptidases are also utilized for accelerating the cheese flavor development in EMCs. United States patent 6271013 (Chevalet et al., 2001) reports a method for obtaining aminopeptidases used to modify the properties of Swiss-style hard cheese. It includes *A. niger* culture filtrate containing significantly high aminopeptidase activity and free of endoproteases. The phenylalanine aminopeptidase (AspC) from *A. niger* culture (NRRL 3112) used has been cloned and characterized (Basten et al., 2006). To produce bioactive peptides with antihypertensive activity using whey protein, United States patent 6998259 is filed (Davis et al., 2006). It discloses treatment of whey protein with animal origin enzyme, along with neutral bacterial and fungal protease from Amano Enzyme Inc. (Nagoya, Japan). A protein hydrolysate with ACE-inhibitory activity from the beta-lactoglobulin rich whey protein concentrate is described in patent United States20100093640 (Bonte et al., 2010). The protein concentrate is initially treated with bacterial heat-labile protease followed by addition of a thermolysin.

5.6.3 Patents on novel device and techniques in dairy products

Various innovations are reported in dairy products for reducing the aging time, production time and to enhance functional properties of dairy products. The patent EP 0691074A1 involves an automated technique for the cheese production comprising one cheese-making reactor (Bidino, 1996). It will help in overcoming the drawbacks, improve the drainage process of the curd and can be applied to various variety of chesses. Reducing the aging time for semi-hard and hard cheeses is another novel invention in dairy products which can decrease the cost of final product. Normally, cheese need 7−21 days for aging and will develop specific texture, aroma and taste due to the various metabolic processes taking place. Patent EP 0535268A1 discloses a technique that can remove the aging criteria in the case of mozzarella cheese and still obtaining the required characteristics (Barz & Cremer, 1993). It involves elimination of aging, so 0.8% salt is added to the fresh cheese curd. The prepared mozzarella cheese is cooled in brine and held for 48 hours before comminuted and then finally packaged. Food manufacturers are benefitting by the use of stabilizers and emulsifiers but nowadays consumers prefer food products with less or no emulsifiers and stabilizers. This is a great challenge for commercial food products and industries. A technique for improvement in functional characteristics of a product is reported in United States patent 7947321 B2. It includes modifying the morphological characteristics of the particles to achieve required functional property (Brophy & Brophy, 2011). Similarly, another United States patent 6861080 explains a method for the preparation of cream cheese without a conventional emulsifier (Kent et al., 2005). In includes methodology which can further reduce the particle size of fat components so that the firmness and texture can be maintained.

5.7 Conclusion and future prospects

Fermented dairy products are providing vital components necessary for human nutritional diet. Traditionally, the fermentation process was slow and done by organisms naturally present in milk. Whereas, latest microbiological and molecular biology-based processes result in nutritionally rich fermented milk products developed under controlled conditions. Development of innovative techniques in dairy products and fermentation, as well as scientific proofs available for nutritive value enhancement and human health benefits are leading to inclination of consumer's towards fermented dairy products. Bioghurt, yakult, actimel, etc., are a few cultured dairy products which are an important component of therapeutic and dietetic products. In this area, intense research is required to prepare dairy products where probiotic organisms are added to increase their therapeutic value. *Lactobacillus* and *Bifidobacterium* species are used for commercial products as probiotic culture. For cheese preparation in the dairy industry, milk-clotting enzyme proteases are used. Proteases have also found various important functions in the dairy sector, such as acceleration of cheese maturation, new texture in cheese and enhanced functional characteristics. Various patents are already available dealing with the use of proteases, the modification of enzyme proteases/the microbial strain producing the protease and the production of dairy products with bioactive properties. The major disadvantage is with respect to the use of genetically

engineered/modified food products or ingredients, as they are not yet accepted as per different laws applicable in different countries.

IP management includes various strategies and actions which need to be aligned with both the country's law and internationally followed practices. IP and the related rights to the inventors are totally dependent on need and response of market, time and cost of filing a patent and further commercialization of the product or process developed. Every industry needs to frame and work for its IP policy, the IP management and strategies as different industry have different forms of IPR. IP trends in India's dairy sciences sector are showing important contributions of the National Dairy Research Institute, Karnal, in terms of patents filed and technology transferred to various national and international organizations of repute for advances in process and product. These efforts paved the way for further modifications and technological innovations in dairy sciences to bring better opportunity for dairy entrepreneurs and thus the society. However, there are a few challenges in the area of fermented and modified dairy food products that could be fulfilled by taking care of following research needs:

1. Further study in selection of productive strains in dairy sector along with proper production and handling procedures for achieving desired benefits needs to be studied.
2. Research is needed in dairy industries to analyze the indigenous dairy products and in improving their shelf life and better survival in gut acceptance by the consumers globally.
3. Further research is required for optimal implementation of bacterial starter cultures for production process that will lead to strain selection and process design. The quantitative data obtained will be analyzed for better process control and to reduce the economic losses.
4. The target for future studies is to screen the most potent culture for the fermentation process, selection of suitable carrier media for growth, and technological developments for designing food products containing viable bioactive organisms for human benefits.
5. Public acceptance should increase for genetically engineered cultures used for fermented dairy products or used as ingredient in dairy products. Research regarding safe use of engineered microbes needs strong understanding.

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Chapter 6

Insights into the technological and nutritional aspects of lactic milk drinks: buttermilk

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6.1 Introduction

Based on the fermentation process, a variety of cultured dairy products are formed by the starter culture bacteria which converts milk sugar (lactose) into lactic acid. Starter cultures have been used in initiating the fermentation process long before anything about microbiology was known. Although many fermented foods can be made without a starter culture, the addition of concentrated microorganisms in the form of a starter culture provides a basis for insuring that products are manufactured on a consistent schedule, with consistent product qualities. Along with lactose and caseins, buttermilk is a good source of whey proteins, milk fat globule membrane (MFGM), minerals and lecithin. Bioactive compounds containing MFGM has been demonstrated with anti-tumor and cholesterol lowering properties which in turn inhibits the growth of *Helicobacter pylori* or prevents gastrointestinal infections (Barukčić et al., 2019). However, in today's industrialized civilization, the human diet has vastly changed, their diet is protein rich with high calories and the intake of beneficial bacteria has decreased. This microbiota is not only reduced by lifestyle diseases but also by aging. Therefore, to recover this microbiota, development of probiotics and prebiotics have been proposed (Gareau et al., 2010). Fermented food products such as cheese, yogurt, buttermilk, tofu, kefir, etc. are in focus globally due to their functional and nutritional utility for several health improving benefits. The chemical composition of buttermilk determines the nutritional and flavor properties of the product. Low resource households utilize this product more frequently than other locally made milk-based products such as fermented milk or cottage cheese. The beneficial microbes in these food products traditionally play an important role in the intestinal ecosystem. Aryana and Olson (2017) reported that consumption of various fermented milk products by people in different regions of the world living under primitive sanitary conditions, especially in hot climates, was desirable because their high acidity kept these products safe by destroying pathogenic organisms. The industrialization and advancement in technological aspects improved the production of various dairy-based products but also brought its own cons that cannot be neglected.

6.2 Buttermilk

Natural buttermilk, a fermented drink, is the residual liquid which remains after butter is churned, that is, milk from the butter. It is usually thicker than other milk. It can be sweet or salty and is a very refreshing drink in summer. It is very popular in North India as "Chaach." Compared to other milk, buttermilk is thicker in texture. According to Niamsiri and Batt (2009), the production of lactic acid in the milk leads to a reduction in the pH level as a result of which casein, the primary protein in milk, gets solidified and makes the buttermilk more acidic in nature. The pH scale ranges from 0 to 14, with 0 being the most acidic. Cow's milk has a pH of 6.7−6.9, compared with 4.4−4.8 for buttermilk. Due to the presence of acid in the milk, the buttermilk is tangy in taste. Though the name is somewhat misleading, it has actually no butter. However, buttermilk has a low content of fats and calories and contains residual fragments of the MFGM as well as allied minerals (proteins, phospholipids and sphingolipids) that have been linked to positive health benefits and nutritional aspects (Contarini and Povolo, 2013). It also provides us with vitamins A, E and K, and calcium

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and has a high content of proteins, carbohydrates and potassium, which helps in boosting our immunity and aides digestion.

6.3 The milk fat globule

The fat globules (3%−5% of total fat in bovine whole milk) consist of a triglyceride core surrounded by a thin membrane called the MFGM. Dewettinck et al. (2008) reported that the membrane of the fat globule (approximately 10−20 nm in diameter) acts as an emulsifier, offers protection against enzymatic attack and protects the globules from coalescence and enzymatic degradation. The distribution of the globules corresponds to tiny and spherical droplets, or globules, stabilized in the form of an emulsion (Singh, 2006). Studies conducted by Danthine et al. (2000) reported that the diameter of a milk fat globule varies from 0.1 to 20 μm with an average around 3−5 μm. It ought to be noted that the distribution and size of the globules depends on the breed of cow, stage of lactation and feed. For example, the average size of milk fat globule from Jersey cows' milk is approximately 4.5 μm while it is 3.5 μm for Friesian cows' milk (Singh, 2006). The microstructure and the size of the fat globule are essential for the texture of dairy products such as cheese. During processing, the smaller globules are more resistant to disruption and have a higher ratio of MFGM to triacylglycerides and yields a higher retention of the membrane in cheese curds. However, the moisture of the curd increases due to the high water-holding capacity of the MFGM (Lopez, 2007).

6.3.1 The milk fat globule membrane

The bovine MFGM represents between 2%−6% of the total mass of the fat globule and is composed of a complex mix of proteins, glycoproteins, phospholipids, triglycerides, cholesterol, enzymes and minor constituents (Keenan and Mather, 2006). The protein content of the MFGM varies from 25%−60% depending on the method of extraction. It has been reported that the membrane contains over 40 proteins. Their nomenclature has been clarified by Mather (2000) as follows (major protein only): mucin 1 (MUC1), xanthine dehydrogenase/oxidase (XDH/XO), periodic acid-Schiff III (PAS III), cluster of differentiation (CD36), butyrophilin (BTN), adipophilin (ADPH), periodic acid-Schiff 6/7 (PAS 6/7), and fatty-acid binding protein (FABP). The main characteristics of these proteins are summarized in Table 6.1. In bovine milk, about 50%−60% of the phospholipids are attached to the MFGM (fragmented or not), and they represent 26%−31% of the total lipid concentration of the membrane (McPherson and Kitchen, 1983; Singh, 2006). The lipid composition of the MFGM is presented in Table 6.2. The structure of the MFGM is schematically represented in Fig. 6.1, derived from the models of Danthine et al. (2000), Evers (2004) and Keenan and Mather (2006). The natural MFGM consists of a tri-layer structure. Firstly, there is an inner monolayer that probably covers the intracellular lipid droplets and originates from the endoplasmic reticulum and possibly other intracellular compartments. In this monolayer, the hydrophobic tails of the polar lipids are in contact with the triglyceride-rich core. Secondly, in an outer bilayer that originates from the secretory cell apical plasma membrane, the outermost hydrophilic head groups of the polar lipids are in contact with the aqueous phase of milk. This inner face of the bilayer has an electron-dense proteinaceous coat. Some globules present inclusions called "cytoplasmic crescent" entrained between the inner coat and the outer bilayer membrane (Danthine et al., 2000; Keenan & Mather, 2006).

6.4 Chemical composition and properties of buttermilk

Buttermilk has emulsion and flavor-enhancing abilities, which makes it a key dairy component in several food applications. The composition of sweet and cultured buttermilk is similar to skim milk. Additionally, the composition of whey buttermilk is also similar to whey. But the fat content is high in buttermilk (6%−20%) compared to skim milk (0.3%−0.4%) or whey (Sodini et al., 2006). The chemical composition of buttermilk depends largely on the amount of water added to cream. Sour buttermilk differs from sweet cream buttermilk (SCBM) in respect to its titratable acidity. The titratable acidity is higher in sour buttermilk ($>0.15\%$); it is sometimes more than 1%, whereas, in SCBM it lies between 0.10% and 0.15%. On the other hand, Sodini et al. (2006) reported that natural buttermilk has wider variations in its composition; it varies with milk quality used for the preparation of curd and the amount of water added in between the churning process. Yet, on the average, it consists of total solids (4%), lactose (3%−4%), lactic acid (1.2%), protein (1.3%) and fat (0.8%). Buttermilk contains high amounts of calcium, which contributes significantly to its health benefits. The human body requires 1000 mg of calcium per day. Low-fat buttermilk contains around 28% calcium. Consumption of 500 mL buttermilk can fulfill the calcium requirement of the body. A good quality buttermilk,

TABLE 6.1 Main physical and chemical properties of proteins of the milk fat globule membrane (Cheng et al., 1988; Heid et al., 1996; Hvarregaard et al., 1996; Keenan and Dylewski, 1995; Pallesen et al., 2001; Walstra et al., 2006).

Proteins	Percentage of total protein	Molecular weight (kDa)	pI	-SS- (SH)	T_d (°C)	Role
MUC1	*n.f.*	160–200	< 4.5	0 (0)	*n.f.*	Protective effect against physical damage and rotavirus
XDH/ XO	20	150	7.7	11 (38)	< 60	Antimicrobial function (gut)
PAS III	5	95–100	n.f.	n.f.	n.f.	unknown
CD36	5	76–78	*n.f.*	3 (0)	*n.f.*	Scavenger receptor
BTN	20 to 43	67	5.0–5.4	1 (0)	58	*unknown*
ADPH	*n.f.*	52	7.5–7.8	*n.f.*	*n.f.*	Possible mediator for lipid-protein interactions
PAS 6/7	*n.f.*	47–52	5.6–7.6	9 (0)	> 80	*unknown*
FABP	*n.f.*	13	*n.f.*	*n.f.*	*n.f.*	*unknown*

Source: Cheng, S. G., Koch, U., Brunner, J.R. (1988). Characteristics of putrified cows milk Xanthine oxydase and its submolecular characteristics. *Journal of Dairy Science*, *71*(4), 901–916; Dewettinck, K., Rombaut, R., Thienpont, N., Le, T. T., Messens, K., Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal*, 18, 436–457; Heid, H. S., Schnölzer, M., Keenan, T. W. (1996). Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. *Biochemistry Journal*, 320, 1025–1030; Hvarregaard, J., Andersen, M. K., Berglund, L., Rasmussen, J. T., Petersen, T. E. (1996). Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. *European Journal of Biochemistry*, 240, 628–636; Pallesen, L. T., Andersen, M. H., Nielsen, R. L., Berglund, L., Petersen, T. E., Rasmussen, L. K., Rasmussen, J. T. (2001). Purification of MUC1 from bovine milk-fat globules and characterization of a corresponding full-length cDNA clone. *Journal of Dairy Science*, 84, 2591–2598; Singh, H. (2006). The milk fat globule membrane–A biophysical system for food applications. *Current Opinion in Colloid & Interface Science*, 11, 154–163.

TABLE 6.2 Lipid composition of the milk fat globule membrane.

Constituents	Percentage of Total lipids
Triglycerides	62
Diglycerides	9
Sterols	0.2–2.0
Free fatty acids	0.6–6.0
Phospholipids	26–31

Source: From Keenan, T. W., Dylewski, D. P. (1995). Intracellular origin of milk lipid globules and the nature and structure of the milk fat globule membrane. In: P. F. Fox (ed.) Advanced dairy chemistry: Lipids (vol. 2, pp. 89–130). London: Chapman and Hall; and Walstra, P., Wouters, J. T. M., Geurts, T. J. (2006). Dairy science and technology (pp. 497–512). Boca Raton, FL: CRC Press.

after packaging has a pH 4.5 and possesses a smooth viscous texture when poured. Additionally, buttermilk also boosts protein intake.

6.5 Types of buttermilk

The following gives four types of buttermilk that are produced.

6.5.1 Cultured buttermilk

Cultured buttermilk is a low-acid fermented buttermilk obtained from low-fat or skimmed milk fermented by mesophilic lactic acid bacteria (LAB) used as a starter culture. It is characterized as having a smooth viscous texture and tasting

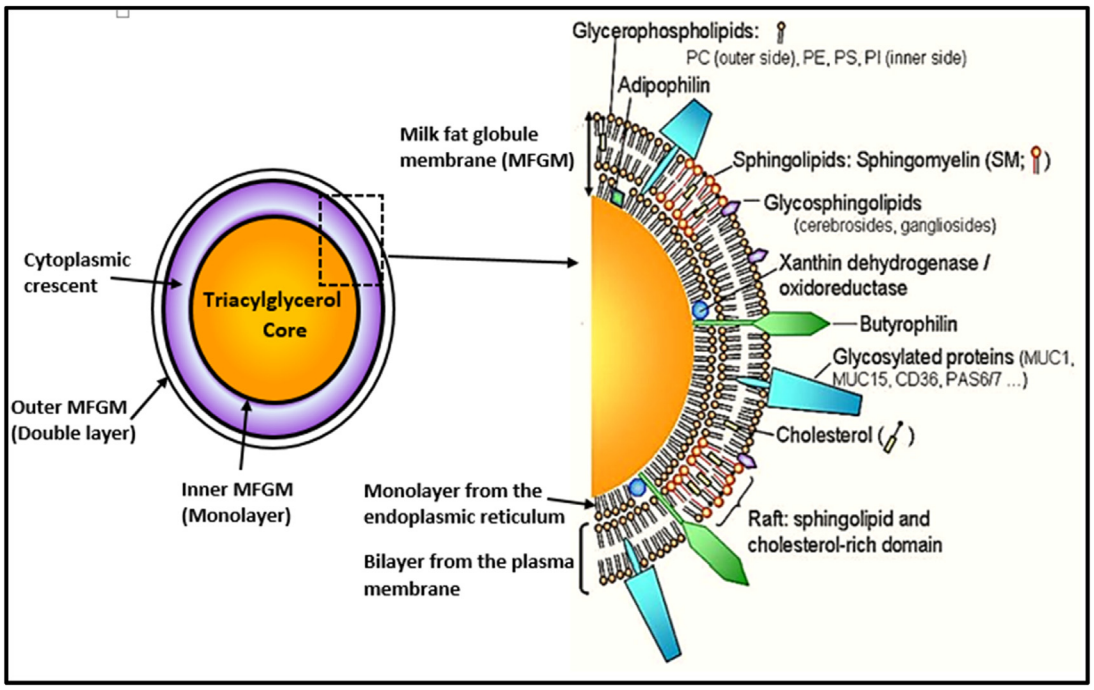

FIGURE 6.1 Schematic representation of milk fat globule membrane with detailed arrangement of the main MFGM proteins. *MFGM*, milk fat globule membrane.

mildly acidic with an aromatic diacetyl flavor. The increased acidity is primarily due to the production of lactic acid as a by-product, naturally produced by lactic acid producers, namely, *Lactococcus lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis*. Whereas, *Lc. lactis* subsp. *lactis* biovar,. *diacetylactis* and *L. mesenteroides* subsp. *cremoris* are the major diacetyl producing microorganisms primarily responsible for the aroma and thus referred to as "aroma producers" (Thomas, 2018). In order to enhance the viscous nature of the product, the milk is heated at a relatively high temperature of 85°C for 30 minutes, thus inhibiting whey-off and disrupting the undesirable microbes and pathogens if present. Upon completion of the fermentation process, the resulting curd is broken down, stirred slowly, and cooled down before being packaged into plastic bottles (Panagiotis & Constatninam, 2014).

6.5.2 Sweet cream buttermilk

Sweet cream buttermilk (SCBM) is produced from churning cream. Churning cream results in the separation of butter and an aqueous phase called SCBM. Generally, cream is not ripened in this case. SCBM has high-fat content as compared to skim milk, which can be decreased by centrifuging or by ultrafiltration (UF) (Conway, 2014). SCBM also consists of huge amounts of proteins, which are drawn by churning from the fat globule-milk serum interface. Apart from their ability to release biologically active peptides (Roesch and Corredig, 2002), these proteins also contribute as a mixture of glycolphospholipids in buttermilk. The phospholipids content in SCBM is around nine times greater than skim milk.

6.5.3 Sour cream buttermilk

Sour cream buttermilk is the dairy product produced by the churning process of ripened cream or milk (Aryana and Olson, 2017). Sour cream buttermilk is not fully fermented and is obtained from raw, unpasteurized sour milk. The milk is allowed to sour naturally prior to churning. It has a tangy, tart taste backed by a rich, thick creaminess.

6.5.4 Commercial buttermilk

Commercial buttermilk does not undergo the churning process for the preparation of milk. It involves the addition of bacterial cultures to skim or low-fat cow's milk followed by maturation. The bacterial culture introduced during the fermentation process gradually thickens the milk, making it more acidic, which in turn imparts its distinctive tart taste.

6.6 Separation, processing and drying of buttermilk

The fat portion of the milk naturally separates from the aqueous phase (skim milk) if the milk is standing for over 30 minutes in a cool place. This phenomenon is usually named "creaming" and is attributed to gravity separation. In fact, milk fat globules are lighter than the plasma phase, and hence rise to form a cream layer (Patton and Keenan, 1975). The rate of rise of spherical particles in a liquid (V) can be estimated using Stokes' Law: /9η where, r = radius of fat globules; d_1 = density of the liquid phase; d_2 = density of the sphere; g = acceleration due to gravity; η = specific viscosity of the liquid phase. Gravity separation is however, slow, inefficient, and hard to reproduce even if Ma and Barbano (2000) demonstrated that the content of fat in raw milk could be reduced to 0.5% after 24 hours of standing at 4°C. SCBM can be used in dry form for various food applications. SCBM is more suitable for processing as it has higher heat stability and its constituent composition is similar to skim milk (O'Connell and Fox, 2000). SCBM remains similar in processes of separation, clarification, pasteurization, concentration and drying at high temperatures. The processes of spray drying and concentration for SCBM are similar to those of skim milk powder (SMP). Spray drying of buttermilk is generally carried out at 185°C−195°C, and the drying process to concentrate the buttermilk is carried out till the 40%−50% solid in end product has been achieved. The major difference between SCBM and SMP is the concentration of total lipid and density. The total lipid consisting of phospholipid content remained a higher bulk in SCBM than SMP, while bulk density was found low. It is generally observed that during storage, high lipid or fat concentration can decrease the shelf life of milk powder. But the high phospholipid content present in SCBM reduces the chances of oxidation in powder.

6.7 Cultured buttermilk

Cultured buttermilk is a naturally produced end product as a result of the fermentation process of pasteurized skim milk, or homogenized low-fat milk, inoculated with cultures of LAB.

6.7.1 Starter cultures used for cultured buttermilk

Microorganisms that are intentionally supplemented into milk for desired fermentation to produce fermented milk products under controlled conditions are called starter cultures. The use of starters has been tremendously important as it decides the quality and nutritional value of the desired end product. But on the other hand, it has diminished the diversity of fermented dairy products (Chawla et al., 2009). Buttermilk starters contain certified organic milk and live active cultures. Large portions of these active cultures (e.g., *Lactococcus, Lactobacillus, Streptococcus*, and *Leuconostoc*) belong to LAB (Table 6.3). Additionally, non-lactic starters can also be used as a co-inoculant with LAB for the production of buttermilk. Starter cultures can be used as single, mixed and multiple strains depending upon the type of products to be prepared for a specific purpose. The purity and activity of starter cultures define their ability to perform functions efficiently. An ideal starter culture should have some characteristics, for example, should be quick and steady in acid production, should produce a product with fine and clean lactic flavor, and should not produce any pigments, gas, off-flavor or bitterness in the finished products. The major role of starter cultures during the fermentation of milk are the production of lactic acid and a few other organic acids, for example, formic acid and acetic acid, changes in body and texture in final products; this is followed by coagulation of milk, production of flavoring compounds, such as diacetyl, acetoin, and acetaldehyde, and production of antibacterial substances in the finished product. Generally, buttermilk products (i.e., sour and cultured buttermilk) are produced by different types of starter cultures. According to Thomas (2018), these cultures are classified on the basis of their temperature and fermentation of glucose for growth purposes, for instance, mesophilic, thermophilic, homofermentative, and heterofermentative bacteria. Products made by use of mesophilic lactic starter cultures (optimal temperature 30°C−40°C) may use one of the following types of microorganisms: O (homofermentative *Lactococcus lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis*), D (microbes of O types and *L. Lactis* subsp. *lactis* (formerly *Streptococcus lactis*) var. *diacetylactis*), L (in addition to the O type bacteria, it contains *Leuconostoc mesenteroides* subsp. *mesenteroides*), and LD (combination of *Str. lactis* subsp. *lactis* var. *diacetylactis* and *Leu. mesenteroides* subsp. *mesenteroides*). Homofermentative bacteria consume or ferment glucose that yields lactic acid as the primary end metabolite. In various dairy culture applications, *Lactococcus* spp. is commonly used as a starter culture, when the quick lactic acid production or low pH is desirable (Hols et al., 1999). Overall, one molecule of glucose (or any six-carbon sugar) is converted to two molecules of lactic acid: $C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$. The sugar fermentative pathway of homofermentative bacteria is shown in Fig. 6.2A. Heterofermentative bacteria also consume glucose; common end products are lactic acid, ethanol and carbon dioxide (Fig. 6.2B). Heterolactic fermentation, where some lactate is further metabolized and results in ethanol and carbon dioxide (via the phosphoketolase pathway), acetate

TABLE 6.3 Microorganisms used as starter cultures for preparation of buttermilk.

Microorganisms	Growth temperature (°C)
Heterofermentative	
Leu. mesenteroides	25
Lb. brevis	30
Lb. kefir	32
Homofermentative	
Lb. lactis subsp. *lactis* biovar. *diacetylactis*	25
Lb. casei	30
Lb. lactis subsp. *cremoris*	30
Lb. lactis subsp. *lactis*	30
Lb. acidophilus	37
Lb. delbueckii subsp. *lactis*	40
Str. thermophilus	40
Lb. helveticus	42
Lb. delbueckii subsp. *bulgaricus*	45

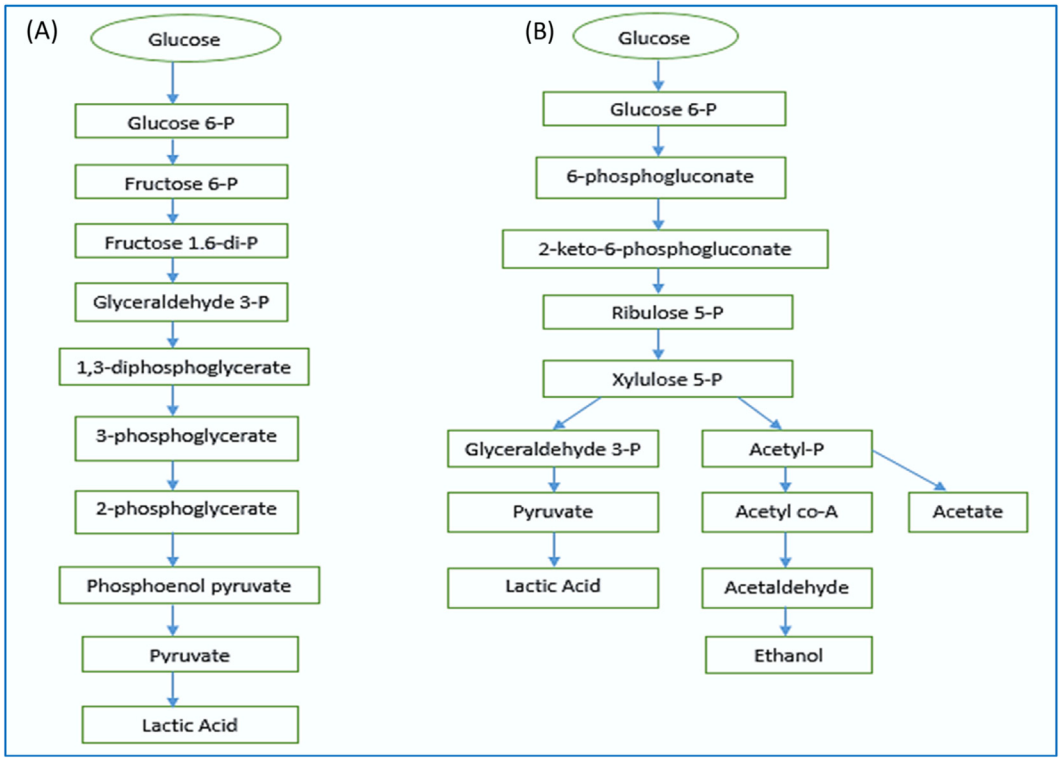

FIGURE 6.2 (A) Homofermentative and (B) heterofermentative pathways of lactic acid bacteria.

or other metabolic products, for example, $C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$. If lactose is fermented (as in yogurts and cheeses), it is first converted into glucose and galactose (both six-carbon sugars with the same atomic formula): $C_{12}H_{22}O_{11} + H_2O \rightarrow 2C_6H_{12}O_6$. The application of heterofermentative LAB as starter culture in dairy products is not common. Yet, these are not rare in dairy products.

6.7.2 Production of cultured buttermilk

It is usual practice to standardize milk for fat and solids-not-fat content looking to legal requirements. Generally, skim or low-fat milk is a starting material for the production of buttermilk. First, the milk is pasteurized at 82°C−85°C for 2−5 min as per need to ensure destruction of potentially harmful pathogens that are present naturally in milk and denature the milk protein to decrease the whey-off. Then, the milk is cooled down to 22°C followed by inoculation with an appropriate mesophilic starter culture (Fig. 6.3). The starter cultures *Lc. lactis* subsp. *cremoris, Leu. citrovorum* and *Leu. dextranicum* are typically used to generate the flavor in butter, while *Lc. lactis* subsp. *lactis* is associated with the production of lactic acid, which produces the typical tangy flavor of cultured buttermilk (Aguirre and Collins, 1993). During fermentation of milk, growth and metabolic activities of LAB cause a few changes in the milk, which results in chemical and physical modifications as shown in Table 6.4. After the buttermilk is fermented sufficiently, it is cooled down rapidly to 5°C−10°C. Next, the coagulum is stirred at a high speed at pH 4−6 until the product becomes smooth. The cooled buttermilk is pumped to the filling machine and filled into bottles or cardboard packs stored at 4°C. The scheme of buttermilk production is shown in Fig. 6.3.

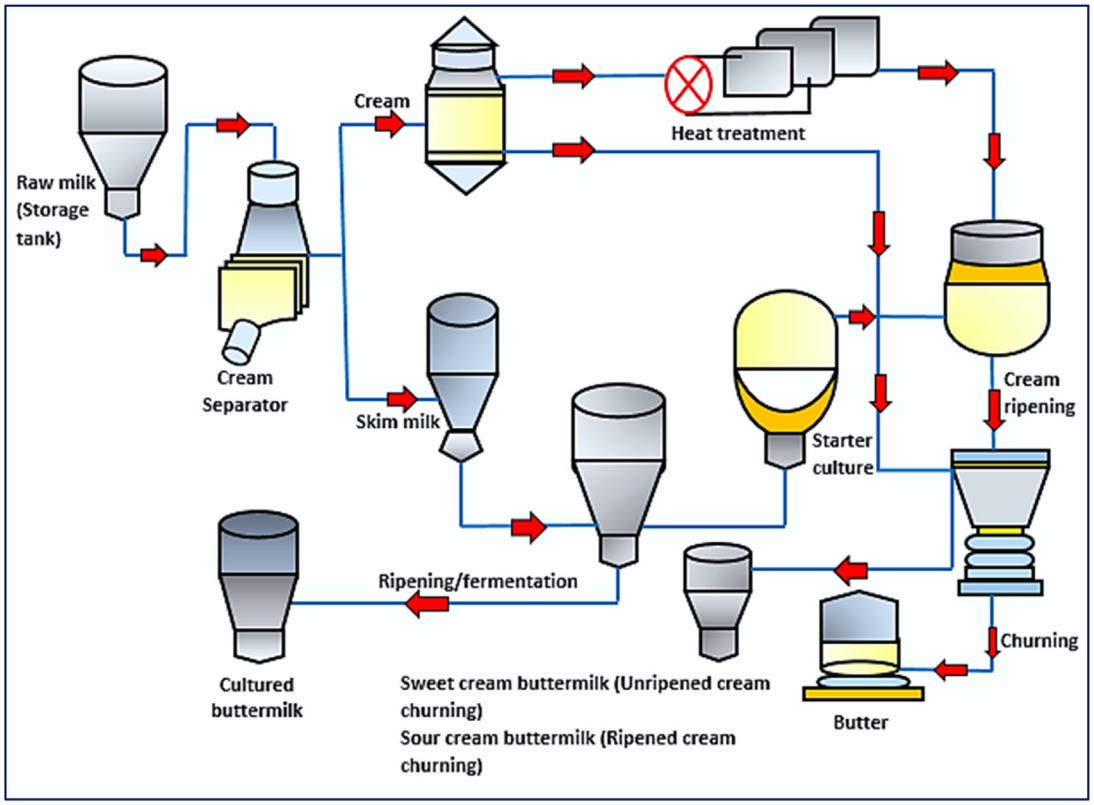

FIGURE 6.3 Large-scale production scheme of buttermilk.

TABLE 6.4 Changes in the constituents during milk fermentation.

Components	Formulation of compounds
Breakdown of fat	Flavored compounds and butyric, propionic, isovaleric, acetic, caprylic, caproic and capric acids generated due to breakdown of fats
Breakdown of protein, for example, casein	Generation of amino acids (serine, glutamic acid, proline, valine, leucine, isoleucine, and tyrosine) due to breakdown of proteins
Breakdown of vitamins, carotenoids, lactose lytic	Simple vitamins, B2, B6, and B12 Lactic acid, galactose, and glucose produced due to breakdown of lactose

6.8 Technological properties of buttermilk

6.8.1 Biofilm formation

Biofilm formation is an important biological concept; whereby, free-floating microorganisms such as bacteria attach to and grow on a wide variety of surfaces to produce extracellular polysaccharides that result in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription. The incorporation, using different proportions of corn starch and buttermilk to obtain biopolymers, as well as the influence of temperature on the polymeric structure, is reported in the literature (Moreno et al., 2014). The study was justified by the application of the biopolymer in sustainable packaging, replacing polymers derived from petroleum and reusing the by-product of the manufacture of butter. The results depicted that the polymers incorporated with buttermilk resulted in separation phases which significantly affected the tensile strength and modulus elasticity. The heating of the films added with buttermilk promoted a positive impact in relation to the resistance, which is justified by the gel formation that, during drying of the film, reduces the critical concentration for its formation.

6.8.2 Production of beverage

A study investigated the use of buttermilk from fresh buffalo milk for the production of carbonated beverages flavored with fresh mango, orange, and pineapple fruit with varying concentrations of fruit juice. The buttermilk used was 0.8% acidity, being first prefiltered to remove casein clots, and then filtered to obtain ultra-filtered buttermilk in Millipore systems. The additional fibers were removed by filtration of fresh fruit juices. Difficulty in the carbonation process was aroused due to the higher content of total solids and low solubilization of CO_2. The use of the fruit juice in the formulation helped to mask the astringent, sweet or sour taste, color, aroma, and palate, as well as the overall appearance and acceptability of the product. The production of the beverage had a higher concentration of vitamins, minerals and proteins compared to the market products as well as better physicochemical properties and, therefore, had a better nutritional quality than the other analyzed samples (Shaikh and Rathi, 2009).

6.8.3 Application of buttermilk in the treatment of industrial surfaces

The procedures for controlling bacterial adhesion on industrial surfaces are of extreme importance, since a short time of contact with the surface is enough for the bacteria to begin biofilm formation. In this sense, buttermilk may aid in the inhibition of the formation of bacterial biofilms on industrial surfaces due to the high concentration of MFGM that possesses polar lipids, which in turn affect the adhesion of bacteria on the industrial surface, preventing the formation of bacterial biofilms (Dat et al., 2014). Some components of bovine milk, such as oligosaccharides, glycolipids and glycoproteins (lactoferrin, immunoglobulins, mucin, etc.) have been reported to inhibit the adhesion of microorganisms—*Lactococcus lactis, Leuconostoc cremoris*, and *Lactobacillus casei*—on stainless steel surfaces for 720 minutes while other products, such as skim milk were able to decrease bacterial adherence for about 30 minutes of exposure, which is considered a short time for the function (Dat et al., 2014).

6.9 Potential health benefits of buttermilk

In Ayurveda, the consumption of buttermilk has been reported to maintain health and as a treatment against diseases. There are reasons behind these uses of buttermilk for health. It is easy to digest, has astringent properties with a distinctive sour taste. It improves digestion and alleviates the feeling of puffiness. It is a natural treatment against swelling, irritation and digestive disorders, gastrointestinal ailments, spleen maladies, anemia and lack of appetite. The ultimate health benefits of buttermilk are discussed below.

6.9.1 Reduces blood pressure

The MFGM present in buttermilk is rich in unique bioactive proteins that have cholesterol-lowering, antiviral, antibacterial and anticancer properties. Potassium in buttermilk also reduces blood pressure. Drinking buttermilk on a daily basis has shown to significantly lower blood pressure and cholesterol.

6.9.2 Buttermilk helps detoxify the body

A good source of many nutrients, buttermilk contains riboflavin, which is responsible for the conversion of consumed food into energy, important in the secretion of hormones and an aid in digestion. The body utilizes riboflavin to regulate and automate the enzymes in the cells, which helps drives energy production. It also aids liver function and assists detoxification of the body. It helps the body make uric acid which is a strong antioxidant.

6.9.3 Potent tool to fight stomach acidity

A powerful drink that combats stomach reflux and acidity, buttermilk has special condiments like spices and ginger that help reduce the burning sensation because of stomach acid reflux or a bout of acidity.

6.9.4 Eases constipation

Buttermilk is a natural therapy often used to comfort the ballooning of the stomach, preventing constipation and other stomach disorders. Incorrect food and timing of meals can put pressure on the digestive system, leading to diarrhea or constipation at times. Regularly consuming buttermilk will ease this condition. Those who suffer from constipation should consume a glass of buttermilk which will facilitate bowel movements.

6.9.5 Strengthens the skeletal frame

Buttermilk, being a rich source of calcium and protein, is an essential building block for the bones and skeletal system of the body. It also helps the teeth become strong. Without any additional calories, buttermilk provides sustenance for new bone development and keeps away osteoporosis. It also provides nourishment to the tissues of the heart and other organs, including nerves and muscles.

6.9.6 Natural therapy against ulcers

Several case studies have been documented to prove that drinking buttermilk is a natural therapy against ulcers. As buttermilk helps neutralize acids in the stomach by coating the stomach lining, it prevents heartburn and keeps the acids from moving up into the esophagus. This drink is effective for people suffering from gastroesophageal reflux disease. Overall, due to of its amazingly refreshing and cooling effect, ulcers too are prevented from erupting.

6.9.7 Treating hemorrhoids

With hemorrhoids, commonly known as piles, being a recurrent problem, ayurvedic home remedies are often recommended along with other medications. If one is suffering from hemorrhoids then adding a cup of buttermilk to a herb mixture herb, particularly chitraka (*Plumbago zeylanica*), could be very beneficial in treating the disorder in a natural way. Consuming buttermilk at least twice daily can regulate bowel movements and helps to get rid of piles problem.

6.10 Advancement in cultured buttermilk technology

With the advent of modernization, several changes and modifications have developed in the manufacture of cultured buttermilk. Based on research, some examples of modifications suggested are given below.

1. Double stage homogenization of milk for 1.7% fat buttermilk adds to richness of flavor.
2. Controlled fermentation process involving the chilling of buttermilk at pH 5.2 to arrest the growth of added *L. lactis* subsp. *lactis* biovar diacetylactis followed by acidification of the product to pH 4.5 with added lactic acid has been reported to give fine flavor through increased diacetyl, but without developing acetaldehyde overtones.
3. By incorporating natural fruits or essences, several kinds of flavored buttermilks can be produced.
4. To improve texture and viscosity of the product, membrane filtration technology can be employed. Concentration of milk by ultrafiltration (UF) can be done to increases protein and decrease lactose content. The process of vacuum de-aeration before cooling is done to remove excess carbon dioxide and helps get better flavor in cultured buttermilk.

6.11 Conclusion

With the development of microbiological and nutritional sciences in the late 19th century came the technology necessary to produce cultured dairy products on an industrial or commercial basis. Nowadays, several functional dairy foods (i.e., probiotic dahi, yogurt, yakult, low cholesterol milk, milk omega-3-milk, etc.) that have a beneficial effect on lifestyle diseases and disorders are very common in the market. Apart from all these products, buttermilk is one of the classical examples of such products. Lactic acid is the main constituent in this drink and has sour taste imparted by LAB, which remains as an integral part of buttermilk even after fermentation. Biochemically, these microbes utilize sugar and yield acids which results in sourness in buttermilk and also leads to a decrease in pH, affecting the casein content, which in turn causes the thickening of milk. Traditional as well as cultured buttermilk has remained an excellent source of nutrition as it is composed of good amounts of potassium, phosphorus, vitamin B12, riboflavin, enzymes, protein and calcium. Buttermilk is also important as it aids in the digestion process and helps maintain water balance and a healthy gastrointestinal tract. Although the health benefits of fermented milk products are great, research is still going on to investigate the role of buttermilk as a potential health benefit that is either indigenous or commercial. To enhance buttermilk quality and health attributes, the appropriate selection of microbes, production strategies and suitable storage protocols have to be adopted or developed.

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Chapter 7

Advancement in acidophilus milk production technology

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7.1 Introduction

7.1.1 Historical background

Nowadays, fermented dairy products have been gaining importance for their functional properties and health-promoting aspects, called functional food or nutraceutical (Granato et al., 2010). It was recommended to have milk fermented with *Lactobacilli* for numerous health benefits and prolonged life in early times. Eli Metchnikoff mentioned in his theory that fermented milk suppresses harmful microorganisms that would produce toxins (Metchnikoff, 1908). We know most people cannot digest lactose; the defect is known as lactose intolerance and can be suppressed by using fermented milk with *L. acidophilus*. The lactose content is reduced substantially during the fermentation process (Gallagher et al., 1974; Kongo & Malcata, 2016). Fermented products are also termed as probiotics. Probiotics are the products that contain live microbes in an amount which, when administered in sufficient quantity, confers health benefits to the host (Hill et al., 2014).

Until 2003, the functional foods primary market was Japan alone and was then followed by Brazil and South Korea. Even other countries such as Germany, Denmark, and France in 1944 also started competing in this market (Lievore et al., 2015; Tian et al., 2017). The world market of probiotics is expected to reach 12 billion dollars by 2026. Many studies have reported the evidence of health benefits of acidophilus milk intake. The health benefits the host experiences includes control of serum cholesterol levels in the body, avoids pathogenic infections of intestine, chemotherapeutic effects against colon cancer, and increase lactase activity among lactose maldigestion patients, effectiveness in reducing inflammation and help in better absorption of vitamins and minerals (Markowiak & Śliżewska, 2017). The fermented milk should be palatable and loved enough by consumers to buy; that's why manufacturers generally add flavors and additives (La Torre et al., 2003). The fermentation is a method that will increase the nutritive value and health benefit of any milk product. Acidophilus milk's health benefits are mainly attributed to its lipids, proteins, vitamins, and mineral composition (Borresen et al., 2012).

7.1.2 Milk-based beverages

Processed milk products which are made by using whole, skimmed, or powdered milk known as dairy or milk-based beverages. Generally, additives such as flavors, colors, fruits, functional ingredients, acids, and class 1 and 2 preservatives are added to these products to enhance sensory attributes. Dairy-based beverages can be fermented or nonfermented. Fermented beverages comprise a strong nutritional profile and contain lipids, carbohydrates, proteins, vitamins, minerals, cytokines, and bioactive components like immunoglobulins, hormones, and exopolysaccharides (Baschali et al., 2017). Fig. 7.1 shows the graphical representation of the last 10 years of published research on probiotics in the MEDLINE database. Different kinds of milk-based beverages are available worldwide; some are discussed below:

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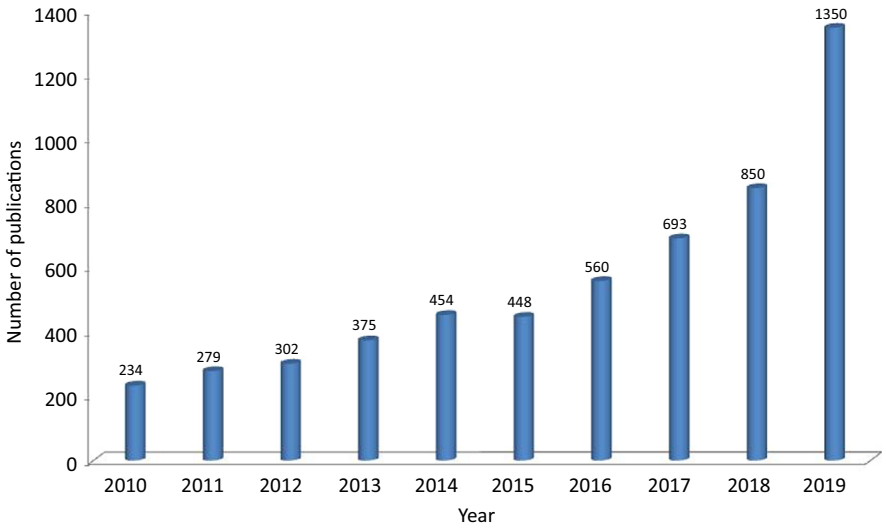

FIGURE 7.1 Number of research published on probiotics in MEDLINE database for past 10 years (2010−19).

Kefir, meaning "good food" in Turkish is manufactured by using kefir grains incorporate into a whole or skim milk. Kefir is fermented by a specific combination of yeast and bacteria, thus imparting slight acidic and carbonated characteristics (Yerlikaya, 2014).

Another milk-based beverage is Koumiss (kumiss), which is manufactured in the Central Asian Steppes, using mare milk and known as "national milk-based beverage." Its nutraceutical uses are weightiness in malnourished patients, energy-boosting, and robustness (Kinik et al., 2000).

In Japan, two dairy-based products are Mill-Mill and Miru-Muru. Mill-Mill is the dairy beverage fermented by the combined starter culture of *Lactobacillus acidophilus, L. casei, Bifidobacterium breve*, and *B. bifidum*. It is a liquid beverage utilized as soup containing carrot juice (source of the precursor of retinol), glucose, and fructose. Miru-Muru is prepared by a combined starter culture of *L. acidophilus, L. casei*, and *B. breve* (Yerlikaya, 2014).

Flavored milk is made by using skim milk or toned milk, sucrose, and additives. Ravindra et al. in 2014, stated improvement in the shelf life of flavored milk by carbonation. Pasteurized flavored milk can sustain 4−5 days shelf life in refrigeration conditions, while the sterilized product can sustain 3−6 months shelf life at ambient temperatures (Ravindra et al., 2014).

7.2 Varieties of milk used in fermentation

Various kinds of milk can influence the flavor, nutritional profile, and smoothness of the end product due to variable chemical composition. Cow milk is an ample source of nutrients and energy due to its good quality lipid profile, that is, linoleic acid, oleic acid, ώ-3 fatty acids, vitamins, and minerals, which impart various health benefits to consumers (Haug et al., 2007). Cow milk, owing to its plenty and low price, is used in most fermented milk-based beverages (Wang et al., 2017). Though, in 2%−3% of the pediatric population, cow milk allergy has been reported, which is common in children (Lifschitz & Szajewska, 2015) and a matter of concern, necessitating early allergy testing.

On the other hand, goat milk possesses less allergenicity, small fat globules, and good digestibility than other milk and, therefore can be utilized to get rid of such constraints. Studies show the therapeutic benefits of fermented goat milk, like anti cardiovascular disease, antimicrobial effects, and antioxidative effects (dos Santos et al., 2017). However, the lower buffer capacity of goat milk means it quickly gets high-acidified and leads to undesirable flavor; thus its utilization for acidophilus milk manufacturing is still a challenge (Alhaj & Kanekanian, 2014; Martín-Diana et al., 2003).

Unlike cow milk, camel milk possesses higher nutrient content such as moisture, ascorbic acid, iron, and amino-acids, except lactose, which is lowest in percentage, i.e. 4.2% (Saljooghi et al., 2017). Coconut milk is plant-based milk, which is widely consumed due to its calcium and phosphorus content. It has a creamy texture and good taste. However, the coconut taste and flavor could be objectionable for some buyers. Hence, it is advisable to fortify with taste masking flavor ingredients to control disagreeable taste (Manoj Kumar et al., 2018). As studied, on selecting base ingredients for cultured milk preparation, each type of milk has its own merits and demerits in expressions of nutritional profile, flavor, texture, and cost that may decide consumer opinion.

7.3 Ingredients used in production of acidophilus milk

7.3.1 Probiotic cultures

Various lactic acid bacteria (LAB) species are certainly utilized and verify to be probiotic. The European Food Safety Agency has suggested a scheme for a "before-marketing" safety analysis of particular sets of bacteria, resulting with the "Qualified Presumption of Safety" position to lactic acid bacteria species such as *Lactobacillus, Pediococcus, Bifidobacterium, Streptococcus*, and *Leuconostoc* (Kongo & Malcata, 2016). During 1980, cultured products were produced mainly by *Bifidobacterium bifidum, B. longum, B. adolescent, B. breve*, and *B. lactis*. However, currently *Lactobacillus acidophilus, L. rhamnosus, L. bulgaricus*, and *Streptococcus thermophilus* are frequently used as probiotic strains (La Torre et al., 2003).

Cultured acidophilus milk is prepared using strains of lactic acid bacteria (LAB), that is, *Lactobacillus acidophilus*, which commonly resides in the human digestive tract. Health benefits, mainly therapeutics, of acidophilus milk make it attractive to consumers. The acidophilus name indicates that this milk is produced via the *L. acidophilus* strain only, or a combination of other LAB species or even with *Bifidobacteria* species. Nowadays, in the market, many food products are composed of different strains of *L. acidophilus*, *L. rhamnosus*, *L. casei*, and *Bifidobacterium* due to the beneficial effects of these probiotic strains with positive responses from consumers.

7.3.2 Prebiotics

Prebiotic ingredients act as a key component in functional dairy products. These are food ingredients that are nondigestible and help encourage the growth of advantageous microorganisms in the colon and thus help improve the host (Niness, 1999). Synergistic effects known as "synbiotic" come into existence when both prebiotics and probiotics are used in combination. The addition of prebiotics (inulin, oligofructose, etc.) improves the growth of existing probiotic strains. It helps establish probiotics in the colon. Inulin, a prebiotic, is a mixture of the fructose polymer chain and yield oligofructose on enzymatic hydrolysis. Prebiotics are generally classified as dietary fibers, and their role is support in digestion, foam stabilization, stool volumization, and stimulation of *Bifidobacteria*; they are also helpful in the preparation of low fat by replacing fat and carbohydrate, respectively (Jenkins et al., 1999; Martínez-Villaluenga et al., 2006; Zárate et al., 2002).

Another prebiotic, honey, contains oligosaccharides that help increase gastro intestinal health by supporting the development of useful microbiota in the colon. Studies prove that honey improves the activity, survival, and development of *Bifidobacteria* related strains in cultured dairy products (Mehanna et al., 2003; Sanz et al., 2005).

Concerning fruits addition to acidophilus milk, numerous examples were stated like the pulp of unripe banana (starch, vitamins, minerals, and phenolic ingredients) incorporated into acidophilus milk, which promoted *Lactobacillus acidophilus'* growth *Bifidobacteria*, without affecting the sensory attributes of acidophilus milk (Vogado et al., 2018). A one of the byproduct of winery industry is grape pomace with rich source of phenolic components. Incorporation of grape pomace to acidophilus milk helps improve the retention of probiotic strains and is absorbed by colon microflora into active metabolites unveiling antioxidant characteristics (dos Santos et al., 2017).

Incorporation of gluten (wheat protein) flour into acidophilus milk was explored and found to enhance the sustainability and survivability of probiotics strains and moreover, the colon microbiota of celiac disease patients (Speranza et al., 2018).

7.3.3 Additives

Acidophilus milk with the time modified when using different cultures combination, various additives fortification like gums, pectin, sorghum, dietary fibers (inulin), psyllium, fruits (banana, grapes), gluten flour, and nuts (phytonutrients source) have been investigated (Chandan, 1999; Li et al., 2016; Vogado et al., 2018). Table 7.1 states different lactic acid bacteria, fortifying agents, and their resultant organic acids used in acidophilus milk (Barat & Ozcan, 2018; Ismail et al., 2018; Li et al., 2016; Manoj Kumar et al., 2018; Morya, Chandra, & Seelam, 2017; Morya, Chandra, & Thompkinson, 2017; Niamsiri & Batt, 2009; Ozcan et al., 2017). Stated adaptations to acidophilus milk results were increase sustainability, nutritional profile, acidification promotion, and sensory attributes. These quality attributes are achieved without excess cost and meet consumers' health prospects (Chandan, 1999; Li et al., 2016; Vogado et al., 2018). As the lactic acid bacteria (LAB) are essential and required by people, they need an exceptional environment (time and temperature) and nutrients to grow well. Morya et al. in 2017, investigated a probiotic beverage incorporated with sorghum, carboxy-methylcellulose, pectin and whey as additives and was found after fermentation to enhance the

TABLE 7.1 Acidophilus milk associated common lactic acid bacteria, fortifying additives and metabolic end products.

Starter culture	Fortifying additives	Metabolic end products	References
Lactobacillus acidophilus	Onion juice	Lactic acid	Niamsiri and Batt (2009), Li et al. (2016)
L. acidophilus Lactobacillus delbrueckii, Streptococcus thermophilus, and *Bifidobacterium lactis*	Fortified with fruits named: grapes (red), mulberry (black)and cherry	Acetic acid, acetaldehyde and lactic acid	Niamsiri and Batt (2009), Barat and Ozcan (2018)
L. acidophilus Lactobacillus rhamnosus, and *Bifidobacterium*	Added with water chestnut powder	Lactic acid and acetic acid	Niamsiri and Batt (2009), Ozcan et al. (2017)
L. acidophilus and *Lactobacillus fermentum*	Banana fruit pulp and skimmed milk powder (SMP)	Lactic acid	Niamsiri and Batt (2009), Manoj Kumar et al. (2018)
L. acidophilus, S. thermophilus, and *Bifidobacterium* mixed with ABT-5	Fortified with dates and honey	Acetic acid, acetaldehyde and lactic acid	Niamsiri and Batt (2009), Ismail et al. (2018)
L. acidophilus, Lactobacillus casei, and *L. rhamnosus*	Sorghum flour, whey, carboxy-methylcellulose, and pectin	Lactic acid	Niamsiri and Batt (2009), Morya, Chandra, and Seelam (2017), Morya, Chandra, and Thompkinson (2017)

nutritive value and health quality of the product (Barat & Ozcan, 2018; Ismail et al., 2018; Li et al., 2016; Manoj Kumar et al., 2018; Morya, Chandra, & Seelam, 2017; Niamsiri & Batt, 2009; Ozcan et al., 2017). Subsequently, additives to fermented dairy products can improve the sustainability of probiotics strains (Abdollahzadeh et al., 2018).

7.4 Production technology of acidophilus milk

7.4.1 Milk supply

Acidophilus milk can be prepared with various milks, that is, cow milk (whole, toned, and skimmed), buffalo milk (whole, toned, and skimmed), camel milk, goat milk, and coconut milk. The milk which is going to be used in processing should be free from naturally occurring microbes. This can be achieved by sterilizing milk by boiling.

Various milks have different chemical compositions responsible for distinct flavor, texture, and end product nutritional profile. The fermented milk-producing procedure differs according to the types of formulated ingredients employed (Martín-Diana et al., 2004).

7.4.2 Starter culture

Acidophilus milk is generally fermented with predominant probiotic strains *Lactobacillus acidophilus*, though other probiotic additions is also practiced. Studies reveal that fermented dairy products produce bacteriocins with antimicrobial properties to destroy pathogens (Charchoghlyan et al., 2017). Dairy products fermented with a combination of probiotic cultures give more desirable quality attributes than a single strain. Nevertheless, it is essential to study the possibility of interactions between various cultures to stimulate the microbial kinetic parameters and sensory attributes of the end product (Tian et al., 2017). Metabolic pathways of LAB have been deciding that bacteria is homofermentative or heterofermentative. In the anaerobic pathway, LAB ferment carbohydrates (sugars) to only lactic acid known as homofermentative. In heterofermentative, LAB ferments carbohydrates to many products such as lactic acid in low amount, carbon dioxide (CO_2), and alcohol like ethanol (Ayyash et al., 2018).

7.4.3 Temperature control

Temperature plays an important role when we deal with fermented products. *Lactobacillus acidophilus* grows best at 32°C−40°C temperature. It shows less development at low temperatures. However, acid production at a considerable

rate has been found at room temperature. A study of four observations of the sample revealed an increase in acidity, that is, 0.65% at 68°F/24 h (Rice, 1928). For the development of traditional acidophilus milk, raw milk should be boiled at 125°C/15 min or 95°C/60 min (Vedamuthu, 2006). Therefore high-temperature treatment releases the disrupted proteins and peptides in milk and boosts *L. acidophilus* (Hati et al., 2017). High temperature treated milk then is brought down cool to 37°C temperature. It is kept at the same temperature for three to four hours, allowing the propagation of any spores if present. After this, milk is again sterilized to kill all vegetative cells and then followed by homogenization, cooling at 37°C, and inoculation with an active culture of *L. acidophilus*.

7.4.4 Processing

Regarding acidophilus milk, it has been found that many therapeutic benefits in the colon have been proposed by this milk. Whole milk or skim milk can be used to produce acidophilus milk, and it supports the slow development of *L. acidophilus*. First, milk is treated at high temperature (95°C/60 min), inoculated with a starter culture (2%−5%), and incubated at 37°C until proper coagulation occurs. Inoculated milk then maintains until the desired acidity level (approx. 5.5−6.0 pH) is reached without alcohol (Hati et al., 2017). For obtaining the required level of *L. acidophilus*, it has been found that 200 g of frozen starter culture added to 2 L pasteurized milk is effective. (Vedamuthu, 2013).

Acidophilus milk contains acid-loving bacterium (*L. acidophilus*); thus some acidophilus milk achieves a maximum acidity level 1% and nutraceutical purpose, 0.6%−0.7% acidity. Therefore regular stock culture transfer should be practiced in the preparation of acidophilus milk to maintain the inoculum in active form as fermented milk marketed in liquid form. Generally, the fermentation process needs 18−24 h. for complete fermentation. After acidity development there are sufficient viable *L. acidophilus* colony-forming units around, but they start decreasing up to consumption time. Due to a decrease in cell numbers of *L. acidophilus* may increase in incubation time. This problem can be handled by substituting one-fourth of *L. acidophilus* strains with combined cultures of yogurt such as *L. delbrueckii* spp. *bulgaricus* and *S. thermophilus* (Kosikowski & Mistry, 1997; Vedamuthu, 2013).

Studies reported that after proper fermentation in sweet acidophilus milk, the prepared product is cooled down and kept at 7°C temperature and found a viability state at approximately 1 month (Vedamuthu, 2013).

7.4.5 Shelf life

The shelf life of all types of fermented milk products are limited due to continuation of metabolic activity during storage in the cold; the titrated acidity increases, which in turn leads to a drop in the number of living cells (Donkor et al., 2006; Goodarzi et al., 2016; Vasiljevic & Shah, 2008). In 1944, two scientists, Leatherman and Wilster, defined the production procedure of cultured acidophilus milk. The process followed with preheated milk sterilization at 121°C for 15 min, then cooled down to 37°C. It was then inoculated at the rate of 2 oz in 4.5 gallons of milk followed by incubation around 36°C temperature until required lactic acid production. It is then cool down to 10°C−15°C temperature followed with packaging in presterilized bottles. Leatherman and Wilster reported the end product's shelf life was 14 days at room temperature (Mital & Garg, 1992).

Nowadays, high-pressure processing (HPP) treatment plays a vital role in extending fermented dairy product shelf life, thus gaining interest. In this high hydrostatic pressure applied to a product, it can be used alone or in combination with elevated temperature (moderately), resulting in complete or partial destruction or inactivation of microorganisms. Experiments show that 400 MPa HPP for 30 min improved the shelf life of yogurt due to changes in the metabolism of LAB (Reps et al., 2001), though 550 MPa high pressure was found suitable to preserve yogurt well for 28 days (Jankowska et al., 2003). It has been investigated that HPP at 400 MPa for 30 min does not impact any changes in proteins and lipids except inactivation of microorganisms in prepared kefir (Mainville et al., 2001).

Studies suggested that the synersis problem in yogurt (low fat) can be reduced by using a combined treatment of HPP at 400−500 MPa and heat treatment at 85°C for 30 min for skim milk (Harte et al., 2003).

7.5 Characteristics and physiology of *Lactobacillus acidophilus*

The description or characterization of probiotic microorganisms is broadly distributed into two categories. The first category elaborates the desirable physiology of probiotic strains in terms of stability in end products (Shah, 2000), capable of resisting low pH (Azcarate-Peril et al., 2004, 2005), capable of withstanding bile (Khaleghi et al., 2010; Pfeiler & Klaenhammer, 2009; Pfeiler et al., 2007), adherence and sustainable in colon atmosphere (Buck et al., 2005), capable enough to improve lactase enzyme activity (Sanders et al., 1996) and produces antimicrobial constituents (Sanders &

Klaenhammer, 2001; Tabasco et al., 2009). In the second category, feeding studies of probiotics observed and noticed the immune response of the host (Bron et al., 2012), serum cholesterol gradually lowers in the host (Shah, 2007), refining the metabolism of lactose in the host (Gilliland, 1989) and inhibiting and preventing pathogen infection in the host (Wang et al., 2017), preventing the infection of *Clostridium difficile* (Biller et al., 1995).

7.6 Mechanism of flavor development

Flavor has an essential aspect of any food product purchase when we deal with fermented beverages. Each fermented product has its own aura of flavor; acidophilus milk also exhibits initial flavor and flavors generated during fermentation due to enzymatic reactions. Enzymatic degradations of proteolysis, lipolysis, and glycolysis occur during flavor development, and proteolysis is mainly responsible for sensory attributes of cultured milk (Fig. 7.2). Peptides and amino acids are produced by the degradation of milk protein (casein) during proteolysis and are precursors of volatile flavors (lactones, alcohols, esters, etc.) (Smit et al., 2005).

Nonetheless, in acidophilus milk, off-flavor and too much sourness might not be acceptable by consumers; hence, the pH analysis is advisable for rapid testing during acidophilus milk processing. However, the stability of starter culture strains can be judged by the titratable acidity (TA) of the beverage. TA accounts best for evaluating microbe stability in contrast with pH (Morya et al., 2020).

The primary motive during the production of acidophilus milk is to mask the original flavor of milk and improve the fermented flavor of particular milk such as goat milk by adding additives (color, flavor, sweeteners, etc.) or probiotic mixture. In acidophilus milk generally, the predominant culture *L. acidophilus* is used, but now some other cultures like *L. bulgaricus*, *S. thermophilus, L. casei*, etc. are also used in combination. Different metabolism pathways of each microorganism lead to the formation of various compounds followed with a unique flavor production of the end product (Smit et al., 2005).

Organic acids increase during the fermentation process and storage period. They dramatically affect the flavor of fermented dairy products like acidophilus milk. Few examples of organic acids are diacetyl, acetic acid, lactic acid, citric acid, and acetaldehyde and are referred to as significant flavor elements in the development of fermented milk.

The addition of herbal drugs in cultured milk is also practiced, as it contains aromatic volatile sulfur compounds and contributes a distinct flavor and aroma to it. Sulfur rich amino acids are cysteine and methionine, and during fermentation, they release out in the process of catabolism by the action of various probiotic strains (Sreekumar et al., 2009). Acidophilus milk obtains its flavor due to some aromatic volatile sulfur components such as methanethiol, dimethyl disulfide, trimethyl trisulfide, and hydrogen sulfide (Sreekumar et al., 2009). Product aroma does not affect the sulfurous aroma of sulfur-containing components, and they positively contribute to product aroma (Parker, 2015).

The goaty flavor of acidophilus milk, prepared with goat's milk, can be masked by fortifying milk with grape pomace extract. Short-chain fatty acids (SCFAs) of goat milk like capric and caproic acids, and caprylic are responsible for goaty flavor. The addition of grape pomace extract enhances the flavor of the end product (dos Santos et al., 2017; Freire et al., 2017).

Studies reported the addition of onion juice into fermented milk to yield various aromatic compounds such as sulfur-containing compounds, alcohols, ketones, aldehydes, butanoic acids, and hexanoic acids, which are responsible for unique flavors (Li et al., 2016).

Acidophilus milk fortification with a different ratio of honey and dates has also been verified useful with a decrease of saturated fatty acids and simultaneous increase in unsaturated fatty acids (Ismail et al., 2018), indicative of a good lipid profile of fermented milk.

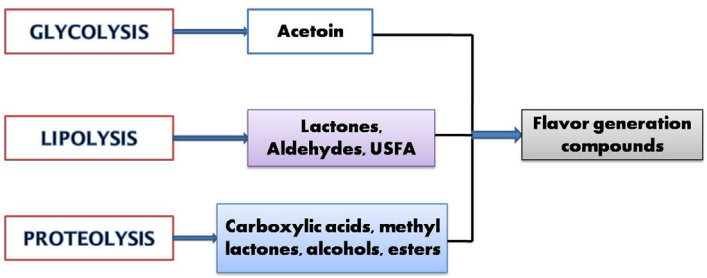

FIGURE 7.2 Schematic of flavor development during fermentation in acidophilus milk.

The formation of acetaldehyde and diacetyl aromatic compounds by adding microelements (zinc) has been found responsible for buttermilk's distinct aroma that increases upon storage (Seleet et al., 2011). Acidophilus milk has a complex flavor profile, which is broadly dependent on the types of milk, ingredients, and kind of culture used for its production.

7.7 Therapeutic benefits of acidophilus milk

7.7.1 Lactose maldigestion

Food intolerance is a common disorder in people according to their body behavior to a particular food, also known as allergy. Among different food intolerants, lactose intolerance is the most common form and happens due to less activity of the stomach's lactase enzyme (Fassio et al., 2018; Misselwitz et al., 2019). Symptoms of lactose intolerance are abdominal pain, cramps, flatulence, vomiting, and diarrhea. These symptoms are caused by colonic fermentation of unabsorbed lactose (Costanzo & Canani, 2018; Deng et al., 2015). The above-mentioned lactose maldigestion symptoms severity fluctuates and varies from person to person. Lactose maldigestion takes place when an individual is not able to digest the lactose. This situation arises when the β-galactosidase enzyme does not exist in a sufficient amount in the small intestine. Fermented products such as yogurt contain β-galactosidase enzyme due to a starter culture; yogurt has benefits against lactose maldigestion. Yogurt culture is supported by the β-galactosidase enzyme and, hence, survives and sustains the intestinal environment, but due to yogurt's acid nature, some people avoid it (Kim & Gilliland, 1983).

Consumption of fermented dairy products (acidophilus milk, yogurt, etc.) has been effective against lactose's maldigestion. Besides, fresh yogurt consumption shows improved lactose absorption instead of a pasteurized one (Saborido & Leis, 2018).

As compared to yogurt, the unfermented acidophilus milk is also advantageous. The starter culture should be grown properly before inoculating the milk that may contain a sufficient amount of β-galactosidase enzyme. The ability of resistance to bile makes it grow well in the small intestine and form extra enzymes. The advantage of unfermented acidophilus milk is that this is not acidic compared to fermented ones (Newcomer et al., 1983).

7.7.2 Anticarcinogenic

A lot of lactic acid bacteria (LAB) are proven to produce antimutagenic and anticancerous characteristics. These characteristics are produced from various compounds during its propagation. They help restrict the conversion of procarcinogen compounds into carcinogen compounds (Hosono et al., 1987). A study was made with rats to check the anticarcinogenic property. The rats were separated into two sets; one group was served with regular milk and milk fermented with *Lactobacillus acidophilus* to another group. The conclusion was that the rats fed with fermented milk had produced some antagonist properties towards cell proliferation (Shahani et al., 1983).

7.7.3 Control of serum cholesterol

For people with hypercholesterolemia, a significant lowering in cholesterol level is seen accompanied by a lowering the risk of a heart attack. It is said that intestinal microflora might affect the serum cholesterol level (Davis et al., 1990). A study was conducted on infants; one group was given formula supplemented with *L. acidophilus* culture. The serum cholesterol levels in the infants who were given *L. acidophilus* were significantly decreased. The decreases in the serum cholesterol level were associated with the increase of lactobacillus obtained in the infant's stool. This suggested that the bacteria acted upon cholesterol in the intestine (Harrison & Peat, 1975).

7.7.4 Resistor of intestinal foodborne pathogens

The *L. acidophilus* strains have been known to show inhibitory actions against the foodborne pathogens. They were conducted in both In-vivo (within a living organism) and In-vitro (in an artificial environment) studies, and also, they show protective or therapeutic effects on colonic infections (Gilliland & Speck, 1977; Hosono, 1977). *L. acidophilus* produces antibiotics like constituents such as acidolin, acidophilin, and lactocidin in the body.

There was also a broad-spectrum antibiotic of *L. acidophilus* produced, which could kill both gram (+) and gram (−) bacteria (Mehta et al., 1983). One of the useful effects of probiotics is the resistance of pathogens because of the

antibiotics; alterations and disruptions in the complex gut micro-flora composition are inhibited. Enough researches have already been conducted on resistance to pathogens activity of probiotics.

Tejero-Sariñena et al. examined probiotics on *Salmonella enterica*, *Serovar typhimurium*, and *Clostridium difficile* in an artificial environment and proposed that probiotics hinder the growth of harmful microbes by the formation of SCFAs (Tejero-Sariñena et al., 2013). Fatty acids like SCFAs support to preserve suitable pH in the colon, which is overbearing in the expression of several bacterial obtained enzymes and in the breakdown of extraneous compounds and carcinogens (Kareem et al., 2014). Several pathogenic resister compounds such as bacteriocins, hydrogen peroxide, alcohol, organic acids, diacetyl, acetaldehydes, and peptides are formed by several probiotic strains (Islam, 2016). Between all these compounds, peptides and bacteriocins, specifically, are frequently participating in growing the membrane porousness of the object cells, resulting in depolarization of the tissue potential, and, eventually, cell death (Simova et al., 2009).

7.7.5 Prevention of *Clostridium difficile* infection

Several randomized control trials of Lactobacillus species with LGG to inhibit *Clostridium difficile* infection (CDI) have been used. In 1983, Drs Sherwood Gorbach and Barry Goldin isolated LGG (a substrain of *Lactobacillus rhamnosus*) from a healthy human's intestinal tract. It has been found that LGG is capable enough to sustain gastric and bile acidity and of inhabiting the digestive tract to employ its probiotic properties (Conway et al., 1987).

Studies reported the antipathogen activity of LGG produces lactic acid, hydrogen peroxide, biosurfactants, and bacteriocins. In the inhibition and treatment of diarrhea, a symptom of several causes of diseases in adults and children, LGG has shown advantageous effects. An open-label study demonstrated that the first case, including five patients with CDI, was cured with LGG administration (Gorbach et al., 1987). In another study, it has been reported that four children suffering from CDI (multiple recurrences) had resolved infection after administration of LGG for 14 days (Biller et al., 1995).

7.8 Conclusions

A crucial commercial bacterium is *L. acidophilus*, which shows a fundamental role in depicting the species *Lactobacillus*. *Lactobacillus acidophilus* frequently ferments milk for the development of various probiotic dairy beverages and products. Fermentation is widely known as a primeval method of biopreservation that is common. Fermented milk beverages have frequently been gaining success commercially. Many lactic acid bacteria (LAB) fermented food products are in the market, such as acidophilus milk, yogurt, unfermented acidophilus milk, yakult, etc. Consumers are attracted to these fermented products from a taste point-of-view and the gain in therapeutic benefits. Fermented dairy products in many studies already showed beneficial effects against many health problems such as lactose intolerance, pathogenicity, anticarcinogen, controlling serum cholesterol, *C. difficile* infection, etc. The continuous increase in consumers' interest in fermented dairy beverages and fermented functional foods indicate that the fermented beverage industries' overall attitude is more encouraging in the present and future.

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Chapter 8

Advancement of yogurt production technology

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8.1 Introduction

Yogurt is a well-known food and is considered an important part of human diets through the ages and has spread and is known as a functional and healthy food (Fisberg & Machado, 2015). Due to the high cost of medical treatment, especially for chronic diseases that have spread in recent times, attention and research have focused on finding foods that have the potential to improve health status and prevent cancer, Alzheimer's and other diseases relating specifically to women's health (Ano et al., 2018; Winblad et al., 2016).

Probiotics are referred to as "live microorganisms," which when administered in adequate amounts extend health benefits to the host (FAO/WHO, 2001). The majority of commercial probiotics are *Lactobacillus* and *Bifidobacteria* species used in products such as yogurt, milk powder and frozen desserts (Shah, 2007; Tamime & Robinson, 2001). It has been known that probiotics have many health benefits such as antimicrobial activity, alleviating diarrhea, anticarcinogenic properties, and improving lactose intolerance and the immune system (Cenci et al., 2002; FAO/WHO, 2001; Shah, 2007). In addition, the medical application such as antibacterial and remineralization effectiveness in nano-toothpastes (Elgamily et al., 2018; Elgamily et al., 2019).

Fermented dairy has gone through many developments and stages. In the beginning, the goal of its manufacture was to save it for a longer period; then its benefits were discovered with increases and developments in its industry, in addition to many compounds and materials added to increase its health and nutritional value (El-Sayed et al., 2017). The additions to yogurt vary, sometimes fruit, herbs and various plant sources rich in fibers, antioxidants, phenols and other compounds are added to fill the shortage of milk, so that the consumer can have an integrated diet rich in all elements (Mohan et al., 2020; Salama et al., 2019; Sarvari et al., 2014; Akl et al., 2020; El-Messery et al., 2021; El-Said et al., 2021). With technological development, ultra-filtration technology has added new products to the yogurt market (Alizadeh et al., 2008; St-Gelais et al., 1992; Valencia et al., 2018). Then the microencapsulation technology emerged for probiotic bacteria and active compounds that are affected by the environmental conditions in the digestive system and during the various manufacturing steps of the product (Joel, 2014; Silva et al., 2018; Salama et al., 2021). The nanotechnology began to appear and shine in the horizons in its various forms to obtain products that have distinctive health properties without any effect on taste, composition or different finished product properties (Chavada, 2016). With the passage of time and technological and industrial development, many technologies will appear, the most important of which is human health. Developments will appear to satisfy his desires to find various products that meet his needs and interests in obtaining a product that has distinctive qualities reflecting his health and activity and protect him from chronic diseases. In this case, it protects him from the high-cost and bad side effects medical treatments are known for. Many studies in recent years have focused on fermented dairy products, mainly yogurt because of its characteristics as a refreshing, healthy drink popular and acceptable to all age groups in different societies. The goal of this chapter is to review the most important developments in the yogurt industry and its health importance, as well as to focus on studies that have concerned women's health.

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8.2 History of yogurt production

The yogurt industry has grown over time and has been the basis for milk fermentation as a means of preserving milk and increasing the shelf life (Weerathilake et al., 2014; Ndife et al., 2014). The origin of the milk fermentation is unclear, although it belongs to civilizations that have been interested in agriculture and animal husbandry such as Samarians, Babylonians, Pharaohs and Indians (Tamime & Robinson, 1999). This was inferred by the discovery of milk fat residues in pottery pots of the Neolithic Bronze Age and Iron Age settlements, which confirm that fermented dairy had existed in Britain for 6500 years (Copley et al., 2003). However, this information is not confirmed, so the onset of fermented dairy is not specifically known. Fisberg and Machado (2015) reviewed the history and origins of yogurt as presented in Fig. 8.1. Aryana and Olson (2017) also review the history of yogurt over the past 100 years.

8.3 Yogurt types

Yogurt is fermented dairy products obtained from fermentation by two species of lactic acid bacteria, mainly *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. This fermentation produces acidification and coagulation, without addition of rennet, and leads to an increase of the shelf life as a result of the low pH (Codex Alimentarius Commission, 2003). Many researchers have looked at the addition of probiotic bacteria as well as the accepted starter culture to increase the health benefits of yogurt. The resulting yogurt with probiotic bacteria is called bio-yogurt or functional yogurt (Yilmaz-Ersan & Kurdal, 2014). Probiotic bacteria are defined as living microorganisms administered in a sufficient number to survive in the intestinal ecosystem, and must have a positive effect on the host (Gismondo et al., 1999).

There are many types of yogurt and it is classified according to many criteria as described in Fig. 8.2; it may exist in the form of set-type or firm, stirred, drinking, frozen, concentrated, or powder yogurts according to texture; natural, sweetened, flavored, or with added pieces of fruits or honey according to added flavors; fat contents and lactose residual content according to shelf life and nutrition (Corrieu & Beal, 2016). There's also the classification that is set according to health benefits.

The different types of yogurt have also been reviewed by Weerathilake et al. (2014). It was according to the physical and chemical nature, as well as added flavors and post incubational processes to be as summarized in Fig. 8.3.

8.4 Raw material for yogurt manufacture

To manufacture yogurt a variety of materials are required, including yogurt, starter, stabilizers, sweeteners materials, fruits and flavorings. Yogurt and yogurt starter are the main ingredients even in the yogurt industry. High-quality milk must be used to obtain good sensory, chemical and microbiological yogurt as per Table 8.1. The choice of the type of milk used in the manufacture of yogurt depends on the type of civet to be produced (Weerathilake et al., 2014).

In addition, yogurt may also be made from nondairy milk or plant milk to meet the needs and requirements of certain groups of consumers in the community (Mäkinen et al., 2016; Tangyu et al., 2019). The most common types of this plant milk is soy milk (Giri & Mangaraj, 2012), Peanut (Rustom et al., 1991), coconut milk (Sanful, 2009; Ndife et al., 2014), coconut and hemp milk (Szparaga et al., 2019), mixed between cow milk and coconut milk (El-Kadi et al., 2017). Other plant sources can be used to make yogurts such as barley milk, rice, almonds, etc. (Desouky et al., 2015; Mäkinen et al., 2016; McClements et al., 2019; Rai et al., 2018; Sethi et al., 2016; Desouky & Salama, 2021).

8.5 Manufacture of yogurt

The main methods of manufacturing yogurt in traditional ways is described in Fig. 8.4. There are many treatments and additions that are added to yogurt, especially if the goal is to manufacture healthy yogurt and those additives are done within the process of manufacturing yogurt in the traditional way. We will review part of these additions in this section. Omega-3, or n-3 polyunsaturated fatty acids, is an important fatty acid known for its health and nutritional benefits for different age groups. Its addition to yogurt is something that enhances its health and nutritional benefits, and increases those benefits if it adds probiotic bacteria. Dal Bello et al. (2015) added five vegetable oils into milk prepared for the manufacture of yogurt before the process of fermentation; these oils are flaxseed, *Camelina sativa*, raspberry, blackcurrant, and *Echium plantagineum*. This study found that the yogurts with added flaxseed and black currant oils were

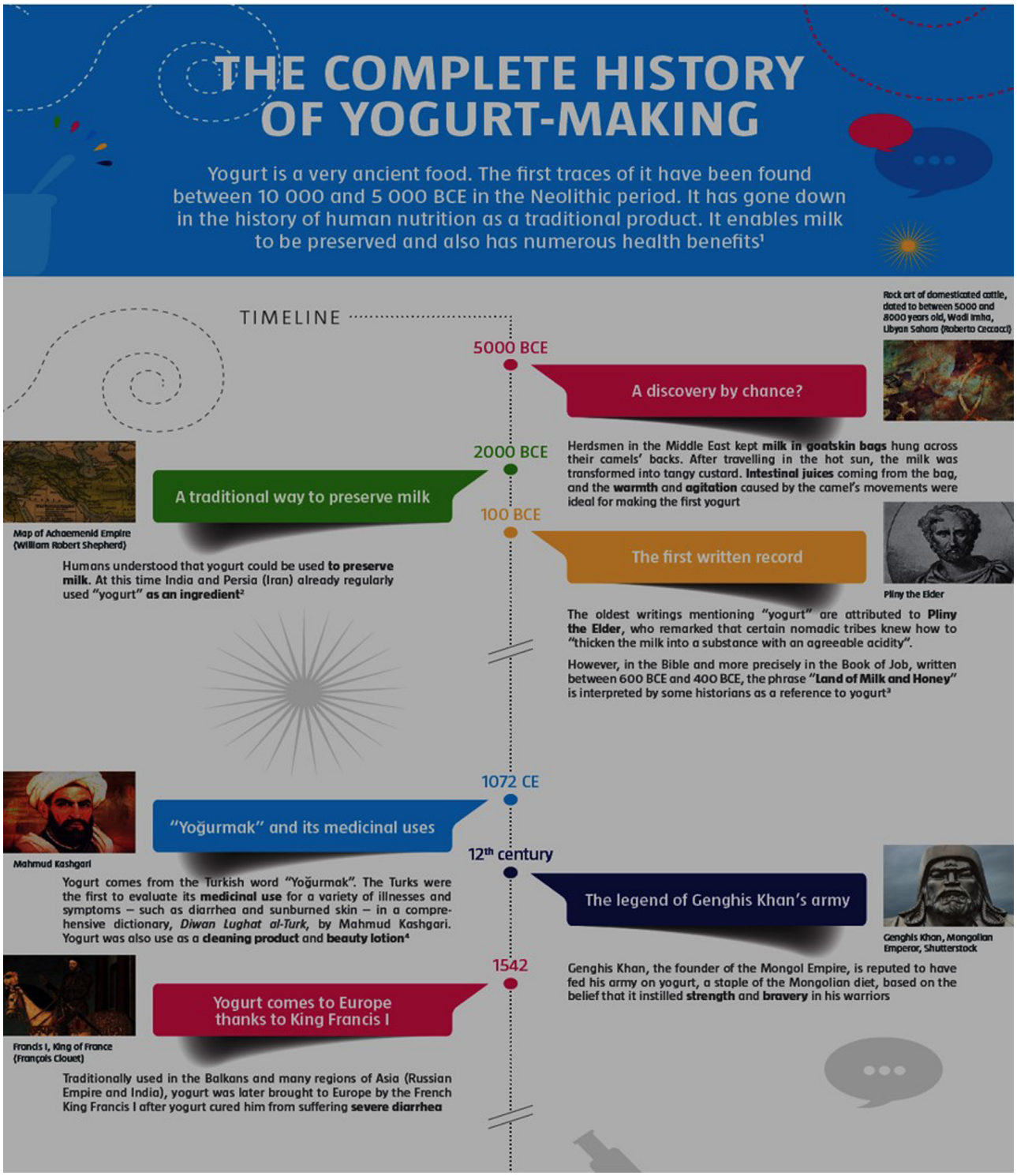

FIGURE 8.1 History of yogurt manufacture (Fisberg & Machado, 2015).

characterized by a high content of α-linolenic acid. Many sources of fiber have been added that lack milk, which has had a good effect on the texture, for example the addition of coconut flour (Salama et al., 2019) as a source of fiber, antioxidants, phenols and probiotics bacteria for bio-yogurt. This is characterized by a distinctive taste and smell that is suitable to be in the school meal for schoolchildren.

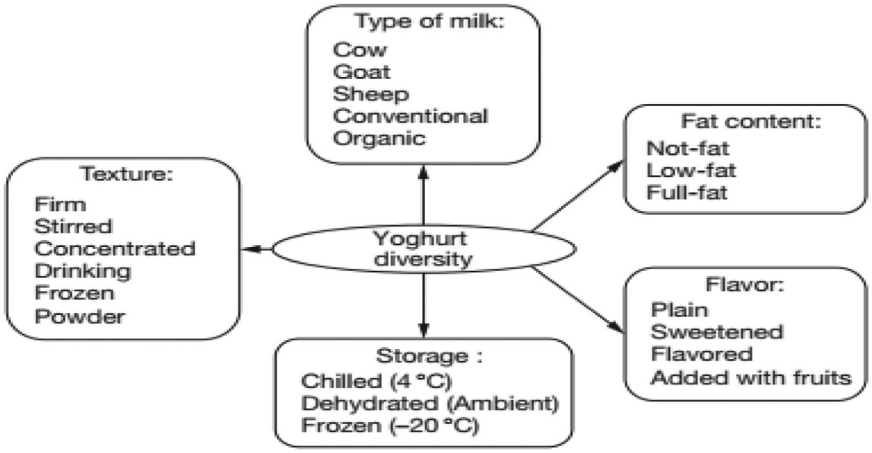

FIGURE 8.2 Yogurt classification (Corrieu & Beal, 2016).

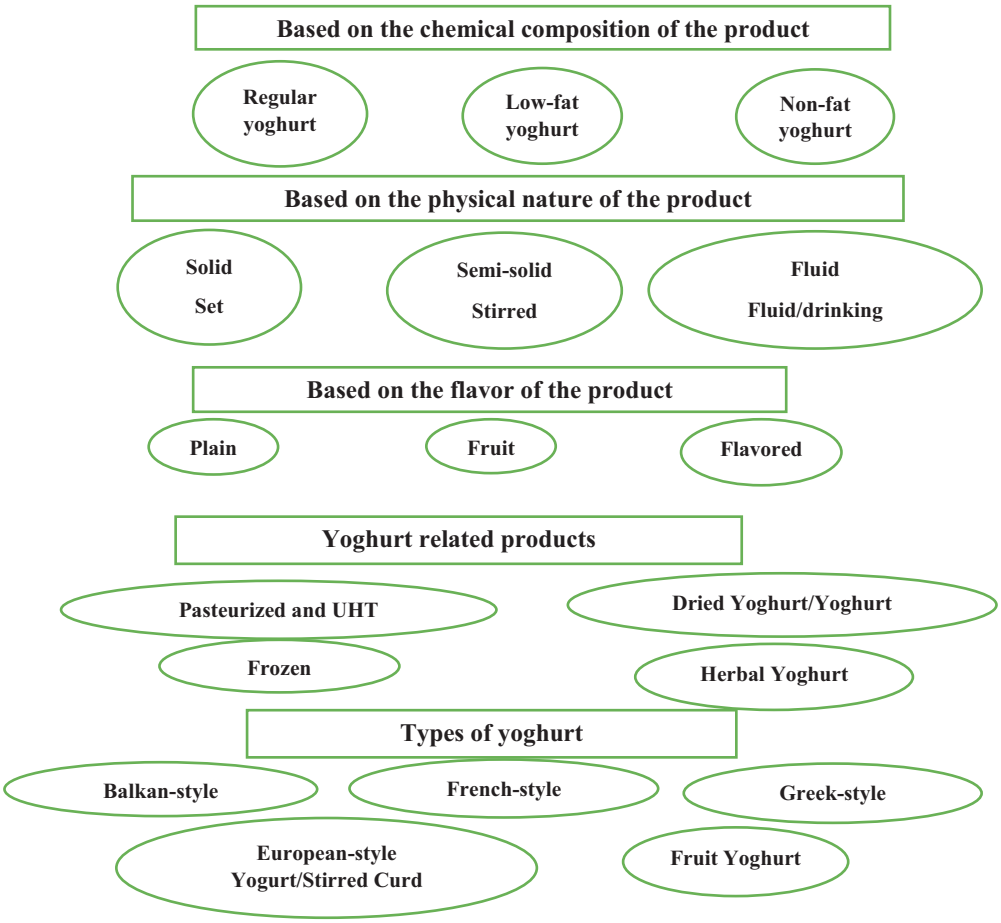

FIGURE 8.3 Different types of yogurt as classified by Weerathilake et al. (2014).

8.6 Health benefits of yogurt

Due to the spread of social media and the availability of internet networks that have made the world a small village, there has been an increase in health and food awareness. The simple consumer has an interest in eating healthy foods

TABLE 8.1 Comparison between cow, goat and sheep milk according to physicochemical properties.

Milk	Fat (%)	Non fat dry extract (%)	Lactose (%)	Protein (%)	Casein (%)	Ash (%)
Goat	4.25–3.80	8.68–8.90	4.08–4.27	2.90–3.52	2.40–2.47	0.79–0.86
Sheep	7.62–7.90	10.33–12	3.70–4.90	5.23–6.21	4.20–5.16	0.90
Cow	3.60–3.90	9.00–9.10	4.70–4.81	3.20–3.50	2.60–2.63	0.70–0.73

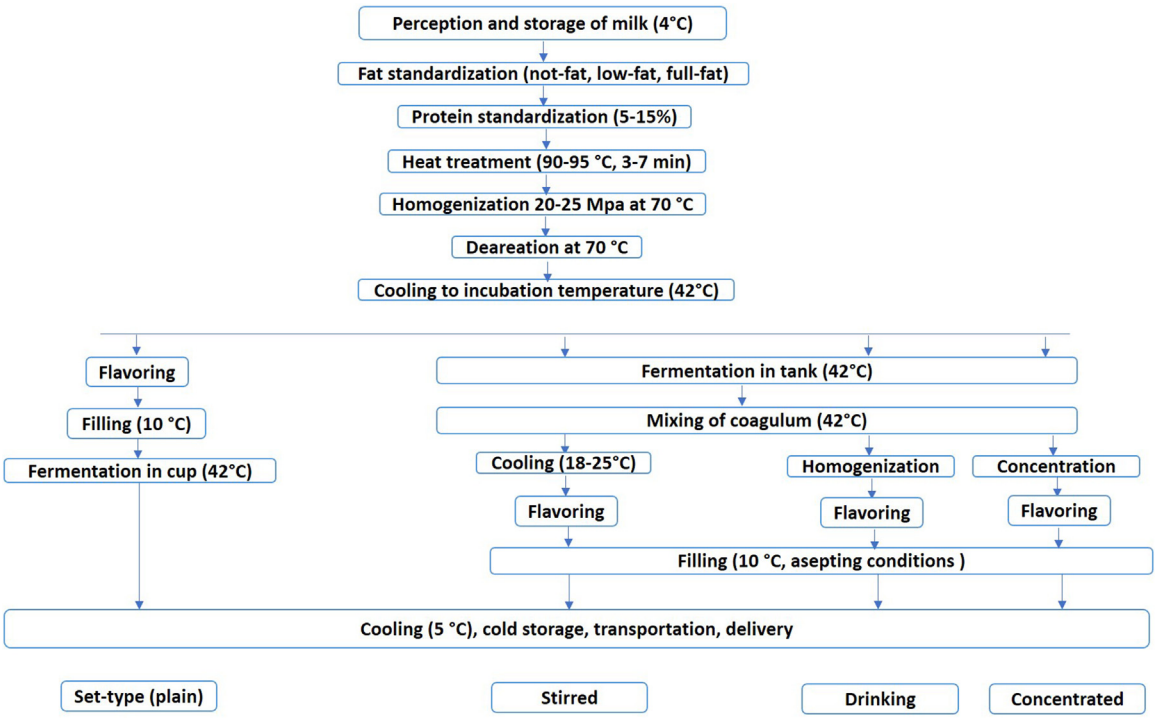

FIGURE 8.4 Main production processes of different type of yogurt (set-type, stirred, drinking, and concentrated yogurts). *Adapted from Corrieu, G., & Béal, C. (2016). Yogurt: The product and its manufacture.* The Encyclopedia of Food and Health, 5, 617–624.

and knowing more of the health and nutritional benefits of what he eats, especially whether he eats them on a daily basis, such as milk and dairy products; the most famous and most important of which is yogurt.

From many scientific studies we can say that there are reasons why yogurt should be taken regularly and daily, which is as follows: Yogurt is one of the most important foods to include in diets, especially in the case of antibiotics, as it is useful to avoid its side effects. Eating yogurt regularly helps the body to produce vitamins, especially vitamin B, which is important for the body's energy balance and protection against neurological and immune diseases (Wang et al., 2013). Yogurt balances sugar in blood as it is slowly absorbed into the intestines (Panahi & Tremblay, 2016). Due to the containment of lactic acid, which distinguishes yogurt from other foods, it provides an environment that prevents the formation of cancer cells and strengthens the immune system. Lactic acid bacteria are bacteria that have important and beneficial effects such as prevention of cancer, digestive diseases and infections. Yogurt, or fermented dairy, is generally considered a disinfectant for the intestines, containing *Lactobacillus bulgaricus* bacteria that increase bowel movement, prevent diarrhea and provide a healthy medium for the intestines. Yogurt is a rich source of conjugated linoleic acid, which has a role in preventing cancer and increasing immunity; it is more useful when obtained from yogurt (Wang et al., 2014). Eating yogurt is a major reason for the prevention of intestinal ulcers and stomach cancer caused by *Helicobacter pylori* infection. That's because the lactic acid kills it and prevents its reproduction. Yogurt eliminates the symptoms of skin allergies by balancing intestinal flora and reducing food sensitivity, which shows symptoms on the skin. Yogurt is also important for women's health. The *Lactobacillus* in yogurt prevents the

production of *Candidal vaginitis* in the vagina. Studies reveal that women who regularly consume yogurt have better vaginal health. Besides all these health benefits mentioned above; yogurt has the same nutritional value as milk and contains the same nutrients (Buttriss, 1997; Gaucheron, 2011; Sahni et al., 2013; Wang et al., 2013). According to the presented and reviewed previously, it is recommended that eating yogurt as a daily meal should be a lifestyle. The detailed of the production processes for different types of yogurt are mentioned in Fig. 8.4.

8.7 New technology for yogurt development

Nanotechnology has been used in recent years to improve the healthy, sensory and technological properties of yogurt (Bajpai et al., 2018). Nanotechnology integrates several disciplines, including physics, chemistry, biotechnology, and engineering, and refers to the use of nanomaterials whose nanoscale structures range from 1 to 100 nm. Nanotechnology focuses on the characterization, fabrication, and manipulation of biological and nonbiological structures smaller than 100 nm. Structures on this scale have been shown to have unique and novel functional properties (Sandoval, 2009). Table 8.2 refers to the different application of nanotechnology in food and dairy products as summarized by Neethirajan and Jayas (2011). And presented in Fig. 8.5 are common applications of nanotechnology in food and dairy products (Nile et al., 2020).

Nanotechnology has been used in the science and technology of dairy for many purposes, including improving the bioavailability of different nutrients (Ahn et al., 2013; Kwak et al., 2014; Park et al., 2007; Seo et al., 2011). For example, loading elements such as iron on milk proteins (whey protein) after preparing them in the form of nanoparticles in different concentrations and characterizing them in preparation for the manufacture of various dairy products. One of this products including yogurt to increase its content of iron, which lacks in dairy and its products. To maintain it and ensure its access to the human bodies with the required concentrations without losing the way by the interaction of other food components or without being affected by different manufacturing transactions or conditions during digestion and absorption.

Loading different fatty acids on milk proteins and cracks is an attempt to form complex among them similar to HAMLET (human alpha-lactalbumin made lethal to tumor cells), which has the ability to kill cancer cells without damaging healthy cells, also for use in the manufacture of different milk products (Salama et al., 2015). Nanoemulsions are also used to deliver important bioactive compounds, such as beta-carotene, that have important health and therapeutic effects (Salama et al., 2016). Frozen yogurt is supplemented by coconut flour nanoparticles prepared with green nanotechnology and probiotic bacteria to increase health properties. Many important ingredients, such as dietary fiber, are lacking in dairy products, antioxidants, phenols and residual fatty acids found in coconut flour. Coconut flour

TABLE 8.2 Different nanotechnology applications for food and dairy industries.

Technology	Description	Benefits
Nanostructures of food ingredients	Nanosized ingredients, additives	Improved texture, flavor, taste; Reduction in the amount of salt and sugar; enhanced bioavailability
Nanoencapsulation of supplements based micelles and liposomes	Delivery systems for supplements	Taste masking; protection from degradation during processing
Nanoparticle form of additives and supplements	Nano-engineered particulate additives	Antimicrobial; health benefits; enhanced bioavailability of nutrients
Improved and active nano-composites, intelligent and smart packaging	Food packaging	Improve flexibility, durability, temperature/moisture stability, barrier properties
Nutrient delivery	Enzymatic structure, modification, emulsion and foams	Targeted delivery of nutrients, increased bioavailability of nutrients
Membrane filtration	Effective separation of target material from food	Higher quality food products and fluids
Surface disinfectant	Engineering nanoparticles	Non-contaminated foods, protection from pathogens
Nanoparticle-based intelligent inks; reactive nanolayers	Nanolithography depositions	Traceability, authentication, prevention of adulteration

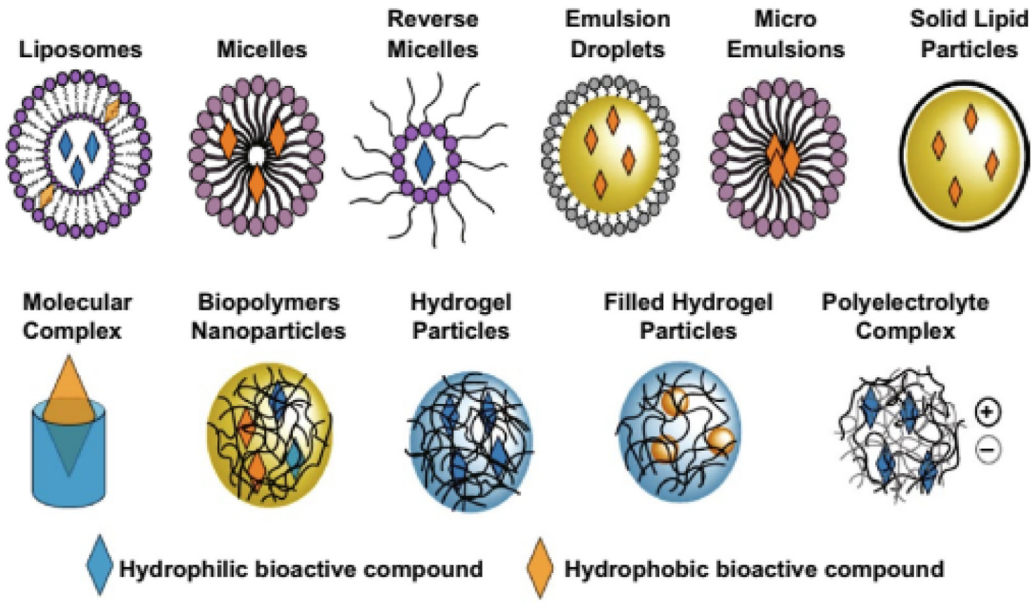

FIGURE 8.5 Common applications of nanotechnology in food and dairy products. *Adapted from Nile, S. H., Baskar, V., Selvaraj, D., Nile, A., Xiao, J., & Kai, G. (2020). Nanotechnologies in food science: Applications, recent trends, and future perspectives.* Nano-Micro Letters*, 12(1). https://doi.org/10.1007/s40820-020-0383-9.*

nanoparticles stimulate the activity of the starter culture as well as the strains of probiotic bacteria and acts as a prebiotic. It was found that its addition to frozen yogurt mixtures in different concentrations, with the presence of probiotic bacteria, increased its activity and improved the sensory and technological properties of the final product (Salama, Abdelhamid, et al., 2020; Salama, El-Said, et al., 2020). Yogurt was added to ethanol sage extract as a good source of phenols after encapsulating them in the form of liposomes. That addition had positive effects on the chemical and rheological properties of the yogurt, as well as the growth of the starter culture and probiotic bacteria (Salama, Abdelhamid, et al., 2020; Salama, El-Said, et al., 2020). Use of nano-powdered peanut sprout in the manufacture of yogurt to increase health benefits, the results of the study by Ahn et al. (2012), showed a decrease in pH compared to peanut powder. Its increased concentration in the nano-image in yogurt resulted in an increase in its antioxidant content (Ahn et al., 2012), while the concentration of no more than 0.1% was sensory acceptable to the consumer and also suitable for microbial growth. It was found that the yogurt made with chitosan nanoparticles powder did not affect the sensory, chemical or rheological, as well as lactic acid bacteria (Seo et al., 2009). The addition of chitosan nanoparticles powder to yogurt aims to improve qualities and properties that are useful in the treatment of certain diseases, which have been confirmed by Park et al. (2010) and Seo et al. (2010). The properties of ginseng improved after it was prepared in the form of nano powder and yogurt manufactured by its supplement it had functional properties and was an active ingredient to obtain functional yogurt (Lee et al., 2013). Egg shells are an unorthodox calcium bumper known for their importance and positive effects on dental and bone health. The preparation of eggshell in nano form improves the bioavailability of calcium as confirmed by studies on experimental animals (Park et al., 2007). The nutritional representation of calcium found in the form of nano powder was better than those in the form of a normal (not nano form). Also, the yogurt made by added egg shells in the form of nano powder. The chemical and sensory properties were not affected compared to those recorded in the form of nano (Mijan et al., 2014; Schaafsma & Beelen, 1999). Santillán-Urquiza et al. (2017) fortified, a set-type yogurt with two levels of iron oxide, zinc oxide, and calcium phosphate nanoparticles.

8.8 Yogurt production technology for health enhancement

Yogurt has many effects on health, especially when it continues to be used on a regular basis. Fig. 8.6 shows the most important health benefits of yogurt content on probiotics bacteria. One of the most important technological developments ever used in the manufacture of yogurt is to have a healthy yogurt that has a clear effect on the diseases of the times, either by preventing the injury or improving the condition and reducing the symptoms associated with the injury. Among the diseases of the age is Alzheimer's disease (AD). The following will review the most important and latest

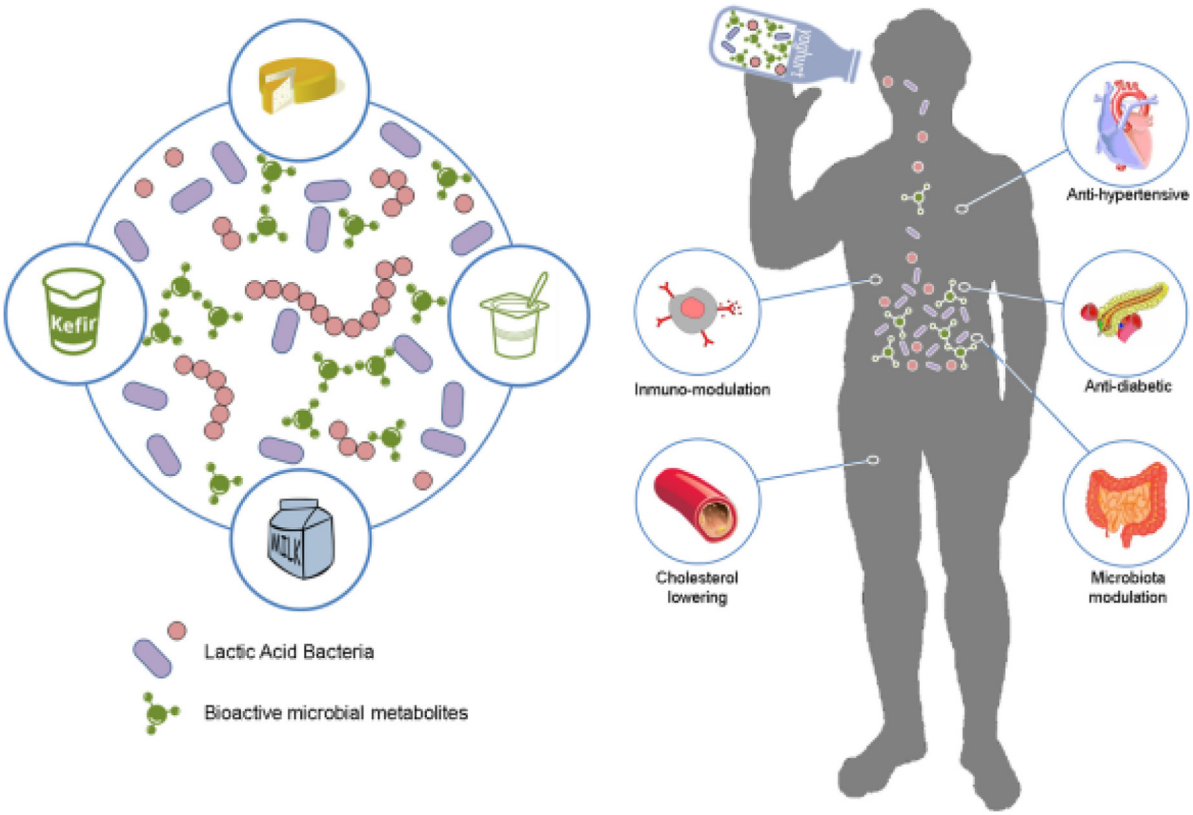

FIGURE 8.6 Main health benefits of lactic acid bacteria (probiotic bacteria). *Adapted from Linares, D. M., Gómez, C., Renes, E., Fresno, J. M., Tornadijo, M. E., Ross, R. P., & Stanton, C. (2017). Lactic acid bacteria and bifidobacteria with potential to design natural biofunctional health-promoting dairy foods.* Frontiers in Microbiology, *8, 846. https://doi.org/10.3389/fmicb.2017.00846.*

studies that focus on the disease, by giving yogurt content on probiotics as an effective means of protection and treatment of the disease (Fig. 8.6) (Linares et al., 2017).

8.9 Application in Alzheimer therapy

Several studies have shown that fermented dairy products have many physiological effects because they contain lactic acid bacteria, fatty acids and peptides produced during fermentation. The fermented dairy products in recent studies have shown that they have effects on cognitive function and a protective role against dementia (Ano et al., 2018). AD is one of the most chronic and slowly progressing neurodegenerative disorders known to date. Researchers put forward the advantages of using lactic acid bacteria (probiotics) possessing antioxidant properties that produces acetyl choline against D-galactose induced AD. This study found that *L. plantarum* MTCC 1325 produce an antioxidant and acetyl cholinesterase has anti-Alzheimer's properties against induced D-galactose because it has led to increased body weight and organ index, improved behavioral activity and learning skills through the rise in the choline neuro transmitter in the areas of the hippocampus and cerebral cortex, and restore histopathological abnormalities support all these preliminary results that have been suggested. So, maybe *L. plantarum* MTCC 1325 has ameliorative effects against AD caused by D-galactose. Another study was carried out to assess the properties of the *L. plantarum* MTCC 1325 strain against D-galactose-induced AD in albino rats. The results of that study showed morphometric and behavioral changes, ACh levels were significantly decreased and pathological hallmarks such as amyloid plaques and tangles were also observed in AD model group. Treatment of an AD-group with *L. plantarum* MTCC 1325 for 60 days, not only ameliorated cognition deficits but also restored ACh and the histopathological features to the control group. However, no significant effects have been observed in the group treated with *L. plantarum* alone. The study revealed that, *L. plantarum* MTCC 1325 might have anti-Alzheimer properties against D-galactose induced AD.

Some studies have summarized that the integration of fermented dairy into the diet reduces the risk of dementia, as confirmed by the Ogata et al. (2016) study, which looked at the relationship between dairy intake and short-term

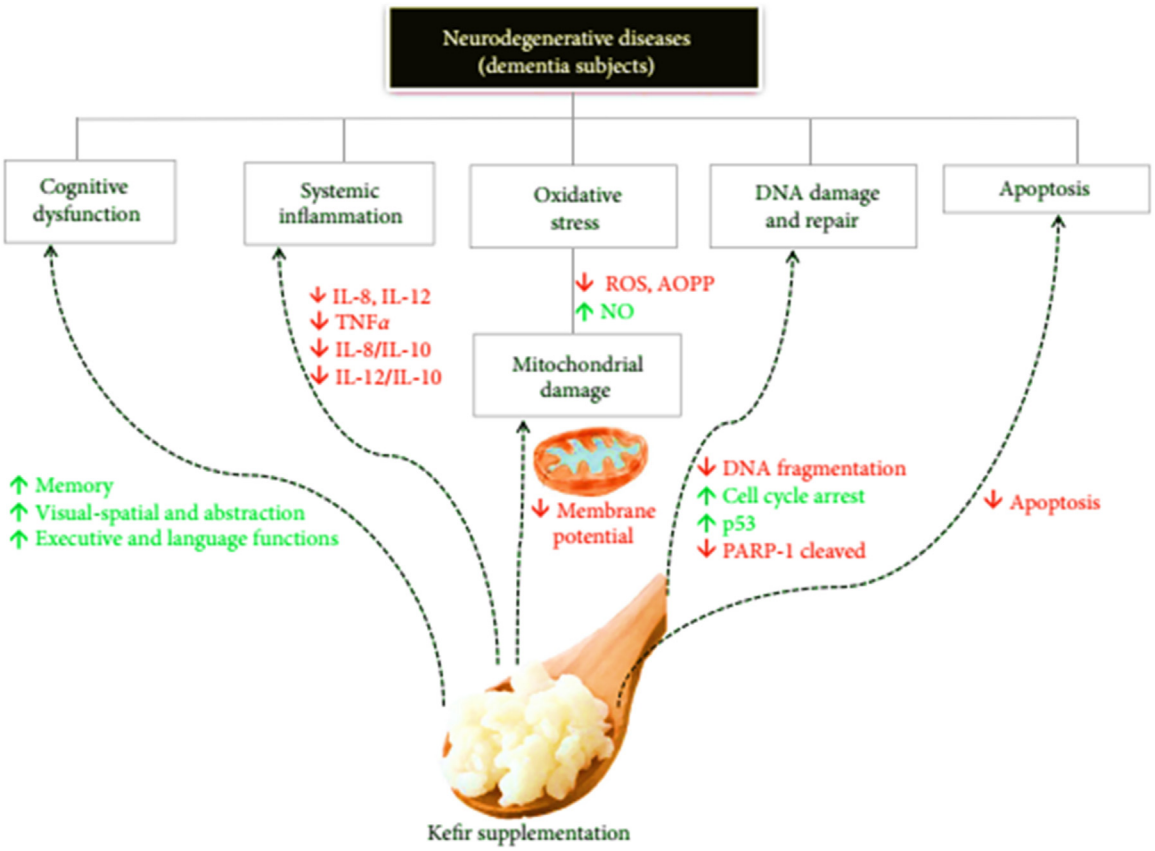

FIGURE 8.7 Beneficial effects of kefir on dementia in AD patients. Simplified scheme of main effects after 90 days with kefir supplementation on Alzheimer's subjects. *AD*, Alzheimer's disease. *Adapted from Ton, A. M. M., Campagnaro, B. P., Alves, G. A., Aires, R., Côco, L. Z., Arpini, C. M., Guerra, E., Oliveira, T., Campos-Toimil, M., Meyrelles, S. S., Pereira, T. M. C., & Vasquez, E. C. (2020). Oxidative stress and dementia in alzheimer's patients: Effects of synbiotic supplementation.* Oxidative Medicine and Cellular Longevity*, 2020, 1–14. https://doi.org/10.1155/2020/2638703.*

memory and found that it is linked to short-term memory. Some studies have linked AD to some deficiencies in metabolic metabolism, and some studies have indicated a link between probiotics and their consumption and cognitive function, where studies on experimental animals have indicated. It was found that the consumption of probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum)* for 12 weeks had a positive effect on cognitive function and metabolism (Akbari et al., 2016). The recent study by Ton et al. (2020) shows the beneficial effects of kefir on dementia patients, which were summarized in Fig. 8.7. The study, which was conducted on the elderly for 90 (2 mL/kg/daily) days by feeding on milk fermented in kefir, showed that it improved the cognitive disability that was associated with three important factors: AD-systemic inflammation, oxidative stress, and blood cell damage, and it can be considered a promising treatment for the progression of AD. Finally, Mustafa et al. (2020) studied the ameliorative role of emulsified yogurt fortified with *ashwagandha* ethanolic extract (AEE) and probiotic bacteria against aluminum chloride ($AlCl_3$)-induced toxicity in rats. The results of this study exposed that AEE and probiotics succeeded to improve physicochemical, microbiological, sensory qualities as well as health benefits as they restored AlCl3-induced hepato-renal-neuro deteriorations; they are a promising supplement for the protection against toxicities Figs. 8.6 and 8.7.

8.10 Women's health

Women are an active member of society, who represents half of society and builds and establishes the other half without which the balance of society is disrupted. Therefore, women's health is important, which is one of the priorities of society, which should receive great attention, especially in developing and poor countries, since in those countries' women incur a lot of burdens that negatively affect their health. Accordingly, we will address in this part the impact of

nutrition on the health of women and their relationship to what they are exposed to by their nature and physical composition.

8.11 Premenstrual syndrome

Premenstrual syndrome (PMS) is a Psycho-Neuro-Endocrine disorder with biological, psychological, and social parameters (Burkman, 2012). Symptoms are divided into two categories: physical and psychological. PMS can be considered as a periodic recurrence of a combination of disruptive, physical, psychological, and behavioral changes during the luteal phase of the menstrual cycle that could interfere with family, social, and occupational activities (Moghadasi et al., 2009). PMS was described by the American Psychiatric Association in 1987 as luteal dysfunctional disorders, and was classified in 1992 along with other symptoms, such as nervousness, as dysfunctional premenstrual in the *Diagnostic and Statistical Manual of Mental Health Disorders* (Bertone-Johnson et al., 2010). Physical symptoms include painful tenderness of the breast, flatulence, abdominal pain, weight gain, edema, headache, back pain, nausea, bowel movements, acne, and psychotic symptoms including irritability, anxiety, nervousness, depression, excessive tiredness and weakness, confusion, changes in mood, sleep pattern, and appetite (Dehnavi et al., 2016, 2017; Jafarirad et al., 2016). Symptoms of PMS may cause many problems, including physical impairment, mental health, and severe functional impairment in women's social and occupational contexts. Symptoms in adolescents may negatively affect their academic performance and their social interactions. Studies have also shown that adolescents with PMS are in poor health (Vichnin et al., 2006). In India, the prevalence of PMS was 18.4%. Moderate to severe PMS was 14.7% and PMDD was 3.7%, according to DSMIV-TR, and 91% according to *International Classification of Diseases*, 10th edition criteria. The symptoms commonly reported were "fatigue/lack of energy," "decrease interest in work," and "anger/irritability." The most common functional impairment item was "school/work efficiency and productivity" (Raval et al., 2016). PMS affects many women during their work life. PMS in working Egyptian women, however, are less well researched. Hammam et al. (2017) found that PMS is highly prevalent among female academic teaching staff in Zagazig University and is more likely to show greater perceptions of impaired work capacity, performance, as well as perceiving work to make symptoms worse.

Yogurt is packed with high amounts of calcium, potassium, protein, phosphorus, magnesium, zinc and vitamin B12. According to a study published in the Nutrition Research in 2013, women who eat yogurt on regular basis have a healthier diet quality as compared to those who don't. As per the study done in 2013, consumption of yogurt on regular basis is associated with lower blood pressure, blood glucose, and triglyceride levels, and less insulin resistance compared with not eating yogurt. This suggests that women who consume yogurt may have a lower chance of developing chronic diseases such as diabetes and heart disease. The colonization of the gastro intestinal tract by microorganisms, known as the gut microbiota, creates an important barrier between the environment and the individual which protects against disease . The gut microbiota can be enhanced when probiotics, live health-promoting organisms, are ingested in sufficient quantities to remain viable after passage through the gastro-intestinal tract (Homayouni et al., 2012). Several species of the *Lactobacillus* genus are known to inhibit the growth of pathogenic bacteria, stimulate immune function, and enhance the bioavailability of food ingredients and minerals, including *L. delbrueckii* subsp. *bulgaricus*, typically used in traditional yogurt. Epidemiologic studies indicate that there are significant associations between yogurt consumption and lower BMI, body weight, weight gain, and body fat, and smaller waist circumference (Eales et al., 2016). Probiotic milk products made with specific bacteria have shown many therapeutic effects in the consumers (Pooja N et al., 2016; Prajapati, 2015). Rai et al. (2018) showed a significant relationship between PMS with respect to life style, BMI and food intake. By proper nutraceuticals, we can reduce the syndrome to some extent. In that regard, the best option is to provide a probiotic yogurt containing *Lactobacillus rhamnosus* and *Lactobacillus helveticus* with conjugated γ-linolenic acid. The probiotic cultures present in the yogurt will help in the reduction of BMI in PMS-affected patients and the γ-linolenic acid will have a therapeutic effect in treating the PMS in women.

Rocha Filho et al. (2011) studied to evaluate the efficacy of fatty acids for the treatment of PMS based on a graded symptom scale and the effect on prolactin levels in plasma and cholesterol. The results of this study showed that there was a significant improvement in symptoms in patients who used the drug containing the active ingredient. Also, using 2 g of this substance, although it led to a higher clinical response, did not alter the end effect of treatment.

It was found that 16 women took capsules containing probiotic bacteria (*L. acidophilus and B. bifidum*) three times a day for 2 months to relieve symptoms of the digestive system in addition to an anti-depressive drug 2 weeks before menstruation and another group took only probiotics. These were compared to nine healthy women as a control group and the results showed that some menstrual symptoms were relieved in the majority of women studied, enough to reduce the severity of the menstrual cycle from severe to mild (Bertazzoni Minelli et al., 1996). *Lactobacillus*

plantarum has the potential to grow unsaturated free fatty acids and convert them into linoleic acid and less toxic conjugated fatty acids. Among all six *L. plantarum* strains, the best one was *L. plantarum* 2−3 which showed maximum growth and conversion of linoleic acid (LA) to different metabolites as reported by Aziz et al. (2019).

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Chapter 9

Innovative practices in the development of yogurt with special concern over texture and flavor

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9.1 Introduction

The term "yogurt" comes from the word "jugurt," which refers to any fermented food with an acid taste (Younus et al., 2002). Yogurt is produced traditionally by fermenting milk with microbial communities. It is a popular product that is consumed worldwide and is considered to have high nutritional and therapeutic properties such that it could be regarded as a functional food (Karagul-Yuceer et al., 2001). Functional foods contain probiotics (live bacteria that convey a positive health effects), prebiotics (compounds that support the growth and maintenance of probiotics), and synbiotics (mixture of both probiotics and prebiotics that act synergically). Probiotics are commonly characterized by improving intestinal microbial balance of the host (Champagne et al., 2005).

Previously, industrial yogurt production and improvement was focused on empirical approaches and experience across the entire process without standard operating procedures or identification of measures. However, current processing of yogurt became industrialized and standardized as a commercial production system in the late 20th century, and continuous scientific developments supported commercial activities to produce wide range of products that could meet new health trends and demands for exciting sensory properties. Scientific studies provided new milk products with modern features (probiotic cultures, bioactive compound fortification) that promote human health and have enhanced sensory and textural characteristics. This has led to high consumer demand for the fermented dairy products including yogurt.

Yogurt is a protein-rich, thickened drink, formed by the coagulation of milk protein by naturally dwelling bacteria and prepared with or without the ingredients such as skim milk powder, caseinate, and concentrated whey to achieve desirable consistency for a targeted consumer group. It is generally assumed that at the time of consumption, such beneficial bacteria may be "viable and abundant" (Chandan, 2006), but the live bacteria count in the product varies depending on processing conditions, product properties, and storage conditions. The previously mentioned description forms part of many countries' food legislation, ensuring that the key features of yogurt are preserved and its conventional view is not breached. There are two forms of traditional commercially available yogurt, namely, set and strained types. Frozen and drinking yogurts have also become quite popular in recent years. In the set type, the fermentation process is carried out in containers and the product is not disturbed with further processing during and after the fermentation process. In the strained type, the product is strained through a cheese cloth after the fermentation and the whey is removed, which produces thicker texture and increases the solids contents, including protein content in the final product. The two types produce different textures with the set one producing a continuous gel texture and the strained one forming a viscous, creamy smooth texture (Tamime & Robisons, 2007).

Dahi, or Indian curd, is a refreshing drink that is used throughout the world. Being one of India's traditional drinks, dahi is the most common and widely ingested fermented milk and matches with Western yogurt in terms of production and distribution. The use of dahi in daily activities goes back in time, and it has been used in rituals and is mentioned

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in ancient scriptures. Dahi is an important intermediate for conventional butter and ghee manufacturing and is used as a basis to produce related products such as lassi, chhash, shrikhand, curd rice, and so on. It is often made using traditional culinary recipes at house and can be made from any type of milk including buffalo, cow, and goat milk.

Cow milk, buffalo milk, or a combination of both are commonly used to prepare dahi. The production of dahi is simple and involves boiling the milk, cooling, addition of dahi starter as an inoculant, incubation over night at ambient temperature (approximately 4−6 h) until it acquires a smooth texture. The Indian Food Safety Standards Authority (FSSAI, 2011) describes dahi or curd a semisolid fermented dairy product, produced by souring (organic or otherwise) from pasteurized or boiled milk as a substrate, by lactic acid or other microbial cultures. The requirements for fermented milk products from the Bureau of Indian Standards (BIS) are based on the type of culture used in their preparation, and normally dahi is made from *Lactococcus*. Like dahi, yogurt is a semisolid fermented product formed by the action of a symbiotic combination of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp bulgaricus* cultures, from a standardized milk mix. Yogurt's texture and consistency is completely dependent on the cow breed, milk composition, and processing conditions of the milk. For the additional buttery odor and taste, Leuconostoc bacteria may be used as adjunct species in the preparation of yogurt. Thermophiles, which are commonly used in the production of yogurt, are isolated from sour dahi (Rakesh et al., 2018).

9.2 Health benefits

Persistence of beneficial microorganisms in the gastrointestinal tract is a prerequisite to have the expected favorable effects in fermented dairy products as well as the stability of bioactive compounds generated during fermentation. Probiotics can tolerate the low pH environment in stomach; thus there are no constraints on their beneficial activity in stomach with low acidic environment (Gibson et al., 1989; Lankaputhra & Shah, 1995). Therefore if these obstacles are survived, the cells can colonize and grow to sufficient numbers to generate the host's beneficial impact.

Yogurt is made from milk, which contains protein and other ingredients such as calcium, vitamin B-2, vitamin B-12, potassium, magnesium, and the beneficial microbes, which all contribute to the healthy nature of the probiotic drink.

Desobry-Banon et al. (1999) and Metchnikoff (1908) discussed the advantages of yogurt and other sour milk intended for personal health. Fermented milk has a long shelf life compared to fresh milk (Bakalinsky et al., 1996). Milk contains several important minerals such as calcium, phosphorous, magnesium, and zinc, and has a wide variety of essential micronutrients (Bissonnette & Jeejeebhoy, 1994; Patel et al., 1992). However, the composition of yogurt may vary according to the processing and the type of milk used in the production. A solid nonfat content in the range from 9% to 15% is required for gel-type yogurt product. The quantity of fat can vary depending on milk source, lactation time, and feeding and farming conditions (Robinson, 1994). Adjustments in milk's physical and chemical properties during fermentation are mostly attributed to the acids produced during fermentation. About 20%−30% of lactose of the milk is converted into lactic acid, which enhances the absorption of nutrients in the gastrointestinal systems because of the production of free amino acids (Gilliland, 1991; Mayo, 1993). It has been reported that the digestibility of milk and other dairy products is dependent on the actions of lactic acid bacteria that facilitate the absorption of nitrogen from yogurt proteins more than milk proteins (Gaudichon et al., 1994; 1995). This is attributed to the ease of digestion and breakdown of the protein curd clots in fermented products after consumption than in nonfermented milk. The larger surface area because of the protein network improves the access of proteases and the breakdown through gastrointestinal tract digestive enzymes (Breslaw & Kleyn, 1973). In addition, a delayed gastric emptying rate is correlated with the viscous consistency of yogurt, resulting in an improvement in the response time of the enzyme substrate (Gaudichon et al., 1994; 1995).

Varela-Moreiras et al. (1992) reported that a substantially higher breath H2 excretion was observed after ingestion of milk or pasteurized yogurt than after yogurt intake in an elderly lactose-intolerant population in a study that investigated lactose ingestion from milk, pasteurized yogurt, and yogurt with active living culture in children and elderly population groups. In children with symptomatic lactose malabsorption, yogurt consumption was suggested to increase their lactose tolerance (Bhutta & Hendricks, 1996).

Under hyperlipemic conditions, rats fed diets based on skim milk and skim milk yogurt had higher excretion of maximum neutral sterols attributable to cholesterol intake. Yogurt consumption also improved the absorption of a bacterial metabolite, coprostanol (Navder et al., 1991). Gilliland et al. (1985) have previously shown that under an anaerobic atmosphere and in the presence of bile, some strains of *L. acidophilus* are capable of assimilating cholesterol. This effect of lactic acid bacteria was later confirmed by Tahri et al. (1997), who reported that bifidobacteria was involved in cholesterol assimilation via the formation of tri-hydroxyl conjugated bile salts.

Perdigon et al. (1995) evaluated the impact of yogurt ingestion on the systemic immune response in mice with active lactic acid bacteria and reported that yogurt might inhibit the development of intestinal carcinoma through enhanced

activation of B cells, T lymphocytes, and macrophages secreting immunoglobulin A (i.e., IgA). Individual immune system activation has also been observed. Halpern et al. (1991) reported that after a four-month diet of two cups of yogurt a day, there was an increase in the development of lymphocyte γ interferon in young humans. The development by bacteria used in dairy foods of individual cytokines was studied in vitro and in vivo by Pereyra and Lemonnier (1993). The development of Interleukin-1β and tumor necrosis factor α was induced by *L. bulgaricus* and *S. thermophilus* in 24−48 h, whereas interferon γ was acquired after 48−72 h. It was shown that the membranes, but not their cytoplasm, were necessary for the formation of cytokines. However, in vivo studies (Baharav et al., 2004) suggested that after absorption of sterile milk or yogurt with a number of 10^{-11} active bacteria, no cytokines were generated. Nevertheless, in the yogurt community, the 2′- 5′-A synthetase activity in blood mononuclear cells was found to be 83% higher than in the milk community. Losacco et al. (1994) investigated the effect of yogurt intake on intestinal immunity post colorectal resection in patients with cancer. A daily ration of 500 g of skimmed yogurt for one month was administered to 10 patients aged 44−85 years who received treatment between 1989 and 1992. With activation of CD4+ and CD8+ cells, yogurt induced a greater release of γ-interferon (Desobry-Banon et al., 1999).

9.3 Functional properties

Yogurt adds a soft, creamy, and sour taste to baked goods, making it suitable for cakes, muffins, and breads to make low-fat choices. Yogurt is also an excellent base for salad dressings and offers creaminess and acidity, without the fat frequently present in oil-based salad dressings. The taste and versatility of yogurt also aid their use in soups and sauce. As a basis for soups and stews, many cultures use yogurt because its creamy texture and acidic taste make it a versatile base for incorporating savory flavors such as garlic, herbs and other flavorings. In dried form, yogurt can also be used in items such as candy adhesives used in nutritional bars or for seasoning cereal products and pieces of dried fruit. Yogurt powder may be used to incorporate the nutritious advantages of yogurt in a more convenient and shelf-stable form in smoothies or other beverage blends (US Dairy Expert Council, 2009).

9.4 Innovative technologies

The initial manufacture of fermented milk products emerged from the need to increase the shelf life of milk instead of disposing of it (Tamime & Robisons, 2007). Previously, the development of yogurt was focused on information and experimental procedures without standard procedures (Fig. 9.1) or on an analysis of the steps that occur during the whole procedure. In the later 20th century, the large-scale production of yogurt became well regulated (Chandan, 2006).

In the dairy industry, the inactivation of somatic cells and the removal of other solid contaminants in raw milk are achieved by centrifugal force (Tamime & Robisons, 2007). This is normally followed by thermalization, which is carried out by heating milk for 20−30 s at the temperature in the range of 60°C−69°C, to kill heat-sensitive vegetative microbes and partial inactivation of some enzymes. This approach triggers almost no other irreversible alteration of the milk (Walstra et al., 2006). After thermalization, the milk is cooled to <5°C, added with lactic acid bacteria; the purpose of cooling is to regulate the psychrotrophic bacteria development (Tamime & Robisons, 2007). In about 80% of yogurts in the United States, *L. acidophilus* is a probiotic bacterium typically used as starter strain (Hutkins, 2006). *L. delbrueckii* ssp. *bulgaricus* is a typical lactic acid bacterium that is commonly used in the production of the most common forms of fermented milk as a culture media (Hartley & Denariaz, 1993) (Fig. 9.2).

9.4.1 Impact of ultrasound milk process on the texture and flavor of yogurt

Ultrasound (US) is a vibration frequency with a wavelength greater than 20 kHz, which is higher than the upper human hearing range. Since the late 1960s, it has been used as a tool in the food processing industry for washing, tracking, and description of food components. When a high-intensity US (greater than 10 W) is propagated through a solvent, it induces tremendous pressure, temperature, and shear gradients, resulting in cavitation (Demirdöven & Baysal, 2009; Dolatowski et al., 2007). As a result, US is used as a facilitator to homogenize the milk and to reduce the size of milk fat globule membrane (MFGM). The use of US in milk decreases the MFGM diameter to between 0.1 and 0.6 m, according to Wu et al. (2009) and Nguyen and Anema (2010). In addition, Krešić et al. (2008) and Chandrapala et al. (2011) stated that US intervention alters the MFGM composition and structure, resulting in a more effective homogenization effect than traditional approaches.

Madadlou et al. (2009) and Chandrapala et al. (2011) investigated the effects of US on milk proteins. The US treatment changes the secondary structure of milk proteins and induces protein particle aggregation and denaturation

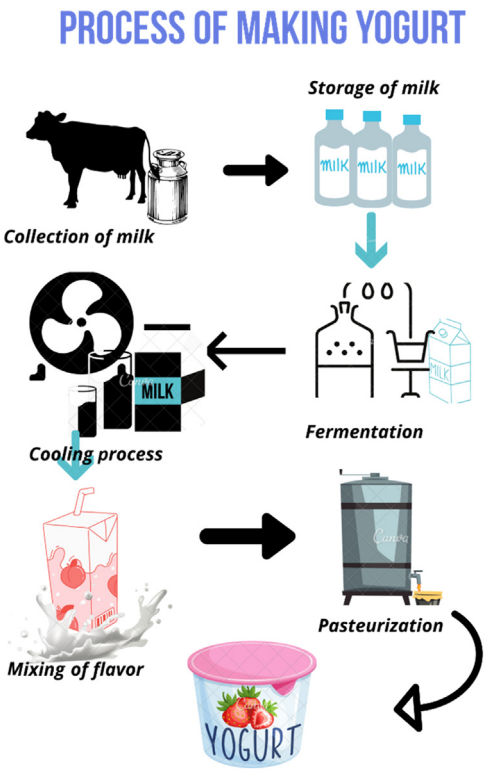

FIGURE 9.1 Process of deriving yogurt from milk.

(Chandrapala et al., 2011; Madadlou et al., 2009). Riener et al. (2009a,b) combined US with thermal processing to obtain the same effect on the MFGM as US treatment alone, resulting in reduction in size and membrane changes that allowed interference with casein micelles. Thermosonication results in an MFGM with an average diameter of 0.6 m and a higher content of casein molecules than the native membrane (Riener et al., 2009a). Additionally, US at high amplitude level reduces the microbial load of milk (Bermúdez-Aguirre et al., 2009; Dolatowski et al., 2007). However, elevated US treatment causes volatiles to escape from milk, leading to the formation of off-flavors. The studies (Riener et al., 2009a,b) indicated that milk subjected to US processing led to the release of benzene, toluene, 1,3-butadiene, 5-methyl-1,3-cyclopentadiene, 1-hexene, 1-octene, 1-nonene, p-xylene, n-hexanal, n-heptanal, 2-butanone, acetone, dimethylsulfide, and chloroform. The formation of aldehydes because of pyrolytic cleavage of fatty acid chains and series of C6−C9 1-alkenes can be formed by the cleavage of hydroperoxides generated by photo-oxidation was triggered by US. The phenylalanine was proposed to be cleaved and cause the formation of benzene. Various positive attributes of US application in fermented dairy products, which include enhanced physical properties, high texture value (firmness, cohesiveness), elevated water holding capacity (20 kHz, 50−500 W, 1−10 min), viscosity, lowered syneresis (Wu et al., 2009), and high denaturation of whey proteins (Riener et al., 2009a,b), may be because of high thermal denaturation of whey proteins. The modification of microstructure of yogurt and high porosity obtained in yogurt by US treatment (Riener et al., 2009a,b) may improve the digestibility of the product. Vercet et al. (2002) reported that combining thermosonication of milk (40°C, 20 kHz for 12 s) with moderate pressure (2 kg/cm^2) added various novel features to yogurt. The treatment of yogurt with US leads to direct consequence, for example, denatured whey proteins linked with casein micelles may act as a bridging material between casein micelles, facilitating the formation of bonds in the yogurt matrix and leading in stronger coagulum (Morand et al., 2011).

9.4.2 Impact of microfluidizing milk on the sensory profile of yogurt

Microfluidizer is a device that uses shear, turbulence, and cavitation to homogenize liquids. It speeds up the fluid and differentiates it into two micro streams that intersect and collide in a chamber. The impact creates a lot of turbulence and cavitation, which achieves the homogenization effect (Ciron et al., 2010; Kasaai et al., 2003). Ciron et al. (2010) found that microfluidization decreased the diameter of the MFGM to less than 2 μm in the case of milk (Skurtys & Aguilera, 2008). Microfluidization has been used in the production of yogurt on numerous occasions. When low-fat

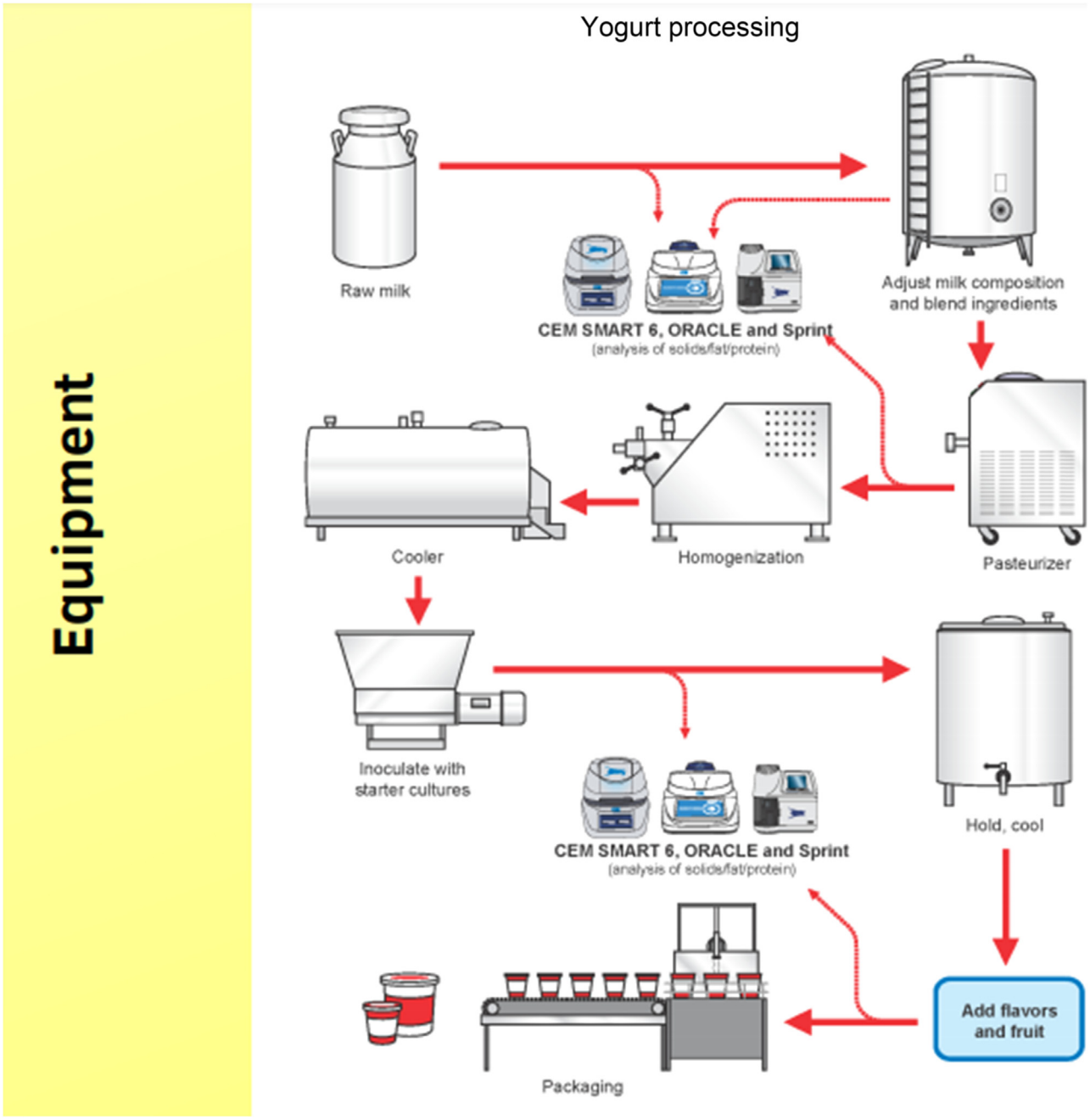

FIGURE 9.2 Modern technologies involved in the production of yogurt.

milk was microfluidized, it produced yogurt with a different microstructure, with more interactions with fat globules but similar texture profiles and fluid retention to yogurt made from homogenized milk. Even so, more research is needed to assess the efficiency of yogurt production using this methodology (Ciron et al., 2010; Ronkart et al., 2010).

Microfluidization at lower pressures may be used to manufacture high-moisture cheese with altered texture, whereas higher pressures may result in novel dairy ingredients (Bucci et al., 2018) (Fig. 9.3).

9.4.3 Impact of ultra-high pressure processing on the texture and flavor of yogurt

The range of pressure from 100 to 1000 MPa, considered to be ultra-high pressure (UHP), is used in dairy industry for the product preparation. UHP is a nonthermal pasteurization method that was first used in food materials in the early

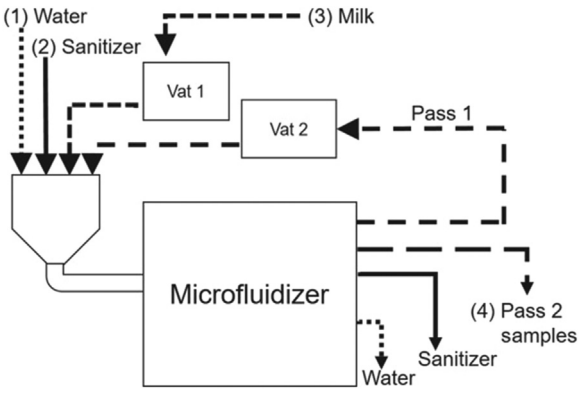

FIGURE 9.3 Use of microfluidizer in yogurt preparation.

1980s. According to several studies, treating milk with pressures in the range of 400−600 MPa for 10 min at 25°C can reach a comparable level of pathogenic and spoilage microorganism inactivation as low-temperature pasteurization (Trujillo et al., 2002). Johnston et al. (1992) and Law et al. (1998) characterized the disintegration of casein micelles into smaller particles and the concurrent increase in the quantity of caseins and calcium phosphate in the serum phase in their studies on UHP treatment of milk. Denaturation of several whey proteins, including α-lactoglobulin and several immunoglobulins and α-lactalbumin proteins, occurs at pressures greater than 500 MPa (Trujillo et al., 2002; Felipe et al., 1997). Although, Gervilla et al. (2001) reported that UHP has an unusual effect on MFGM size and size distribution. At 25°C and 50°C, UHP up to 500 MPa decreased the diameter of MFGM by 1−2 m, but at 4°C, no reduction in diameter was observed. In contrast to traditional yogurts, the use of UHP in the formulation of milk acid gel enhances texture and firmness, decreases syneresis, and improves fluid-holding ability (Trujillo et al., 2002). Ferragut et al. (2000) found that combining UHP and thermal treatment improved yogurt viscosity and decreased gelation times as opposed to UHP control groups (Fig. 9.4).

The food industry is especially interested in nonthermal techniques such as high hydrostatic pressure (HHP). Microorganisms, particularly food-borne pathogens, have been found to be inactivated at pressures ranging from 100 to 1200 MPa. HHP also enhances milk rennet or acid coagulation without affecting the taste, body, texture, or nutrients. Lengthened shelf life and a "fresh-like" product appearance illustrate the value of completely accounting for food safety risks as well as potential customer nutritional benefits. These properties provide the dairy industry with a range of practical applications for producing improved features of microbial products with less processing. As a result, HHP is an important method for developing novel dairy products with enhanced features in nutrition, texture, shelf life, and sensory aspects (Rekha et al., 2011). The compressibility of the pressure medium and the design of the food content affect the time taken to produce pressure in the vessel. The most popular medium for transmitting pressure is water. Since air is much more compact than water, the existence of air in the food lengthens the pressurization time. Isostatic pressure is used to apply the pressure, and the pressure is kept constant throughout the whole process during which the whole product receives the level of treatment (Fig. 9.5). While high pressure is nonthermal in nature, it does cause a slight adiabatic temperature rise (Ohlsson & Bengtsson, 2002).

Syneresis and low viscosity are common defects in yogurt. Pressure treatment may enhance the consistency of yogurt and its rheological properties. HHP (400−500 MPa) and thermal treatment (85°C for 30 min) resulted in high yield, elasticity, and reduced the syneresis in skim milk yogurt (Harte et al., 2003). In a study, when the centrifugal force was applied (1500 g) for 25 min, it increased the whey holding capacity more than 20% whey were retained (Hernández & Harte, 2008). Needs et al. (2000) found that pressure-treated (60 MPa for 15 min) set yogurts had lower fracture stress than heat treated milk. Yogurt prepared with HHP at 200 and 300 MPa at 30°C and 40°C had lower lipid oxidation and lipolysis (Serra et al., 2008).

9.4.4 Role of pulsed electric field in yogurt manufacture

The pulsed electric field (PEF) theory is based on the use of a high-voltage intensity, up to 120 kV, to inactivate microbial cells and introduce changes in the structure of macromolecules. PEFs cause electroporation of the cellular membrane and make it more permeable, causing the cells to rupture and expel their contents (Wouters et al., 2001). PEF treatment can be used in manufacturing yogurt for the purpose of inactivating endogenous undesirable microorganisms and enzymes. PEF was used in conjunction with probiotic cultures (da Cruz et al., 2010). Lin et al. (2002) used a

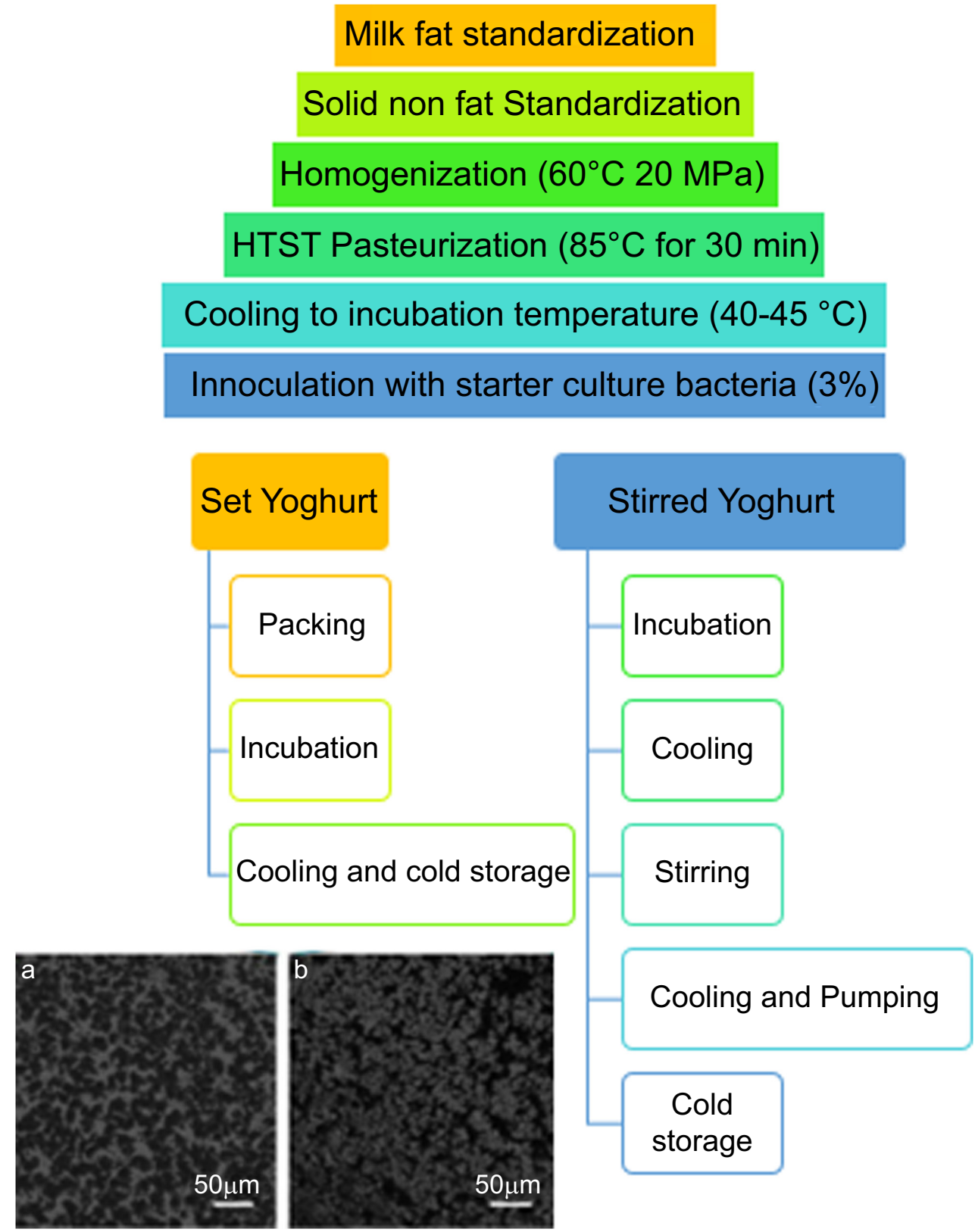

FIGURE 9.4 UHP method of manufacturing yogurt. *UHP*, Ultra-high pressure.

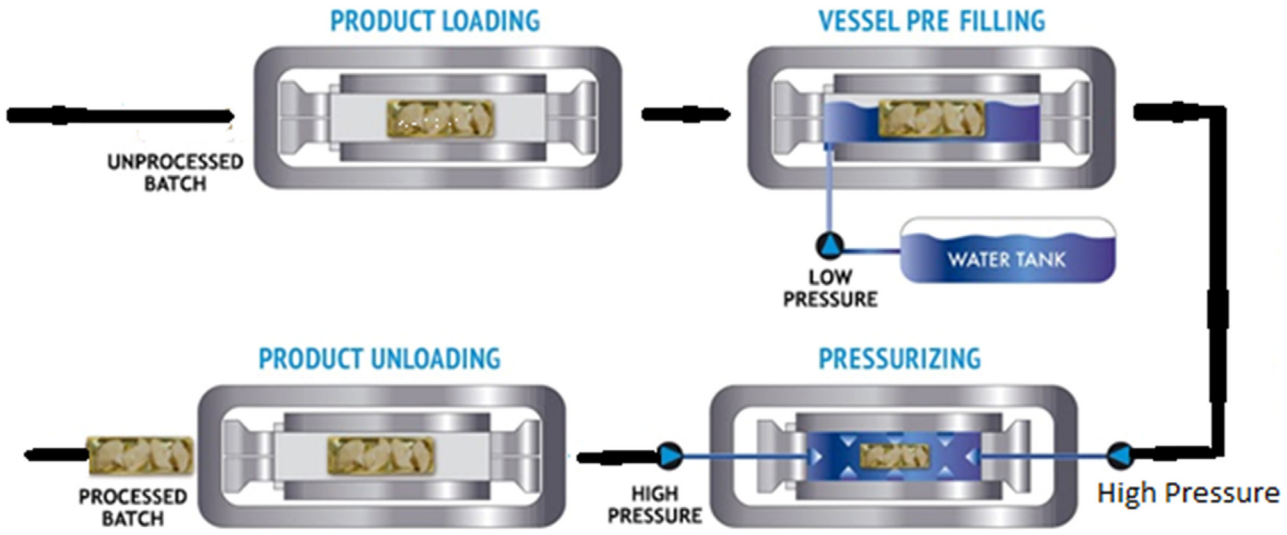

FIGURE 9.5 High-pressure processing of yogurt.

mixture of PEF, UHP, and heat treatment to investigate their effects on milk microbiological quality. The performance of PEF was dependent on the length of pulses and strength of the electric field (Calderón-Miranda et al., 1999). Successful application of PEF requires special attention to processing conditions (pulse shape, PEF strength, and pulse time and frequency), low electric conductivity, and the absence of any entrapped gases (Kelly & Zeece, 2009; Calderón-Miranda et al., 1999).

The PEF treatment system for liquid applications requires a cooling system and a series of cofield tubular treatment compartments, two boron carbide electrodes, and an insulator that acts as the treatment chamber.

PEFs is a new technology that can be used to pasteurize liquid foods, among many other applications. In contrast to pasteurization, PEFs can sterilize foods without increasing their temperature substantially, retaining their sensory attributes. The device operates by delivering pulsing energy between the electrodes of a treatment chamber that contains liquid food (Barbosa-Cánovas & Altunakar, 2006). The propagation of voltage pulses lasting less than 1 s to fluid materials positioned between two electrodes is known as PEF processing. The electrical pulses move through the food to the microbial cell membrane, triggering electroporation and, at higher intensities, irreversible cell membrane damage, intracellular material leakage, and cell death. Microbial inactivation efficacy is determined by microbial characteristics, intervention medium characteristics, and several PEF operational variables (Fig. 9.6). The electric field power, treatment time, pulse frequency, basic energy applied, as well as the geometry and polarity of the pulses are all factors to consider (Alvarez et al., 2006). PEF has been used to pasteurize liquid food effectively in a number of items, in accordance with the quantity of the total energy used (Toepfl et al., 2006). Mild PEF treatment has been shown to increase the efficiency of lactic acid bacteria in experiments (Panagiotis et al., 2020).

9.5 Food additives

Over time, research considering yogurt has been increasingly evolving to achieve greater appeal, improving consistency of the product to fit special needs and nutritional value to meet the increasing and often changing customer preferences and market competition. Mouth feel, taste, and texture are essential sensory attributes of the consistency of yogurt that eventually determine the manufacturer's and consumer acceptance (Kunal et al., 2017). Multiple kinds of additives are used in the manufacture of yogurt, mostly to improve the sensory characteristics. There are distinct characteristics and properties for each category and for each substance from the same community. For this reason, apart from attachment selection, the quantities of the additives are very essential for promoting yogurt sensory characteristics (Milna et al., 2008). Several strategies have been used to enhance yogurt consistency, such as increasing milk solids (adding fat, proteins, or fructose such as sucrose and fructose), and the use of hydrocolloids and stabilizers (pectin, starch, alginate, and gelatin) (Duboc & Mollet, 2001).

Exopolysaccharide (EPS) developed by LAB has received significant interest in the fermented dairy industry. EPS developed by yogurt starter cultures has been documented to influence yogurt texture and improve sensory

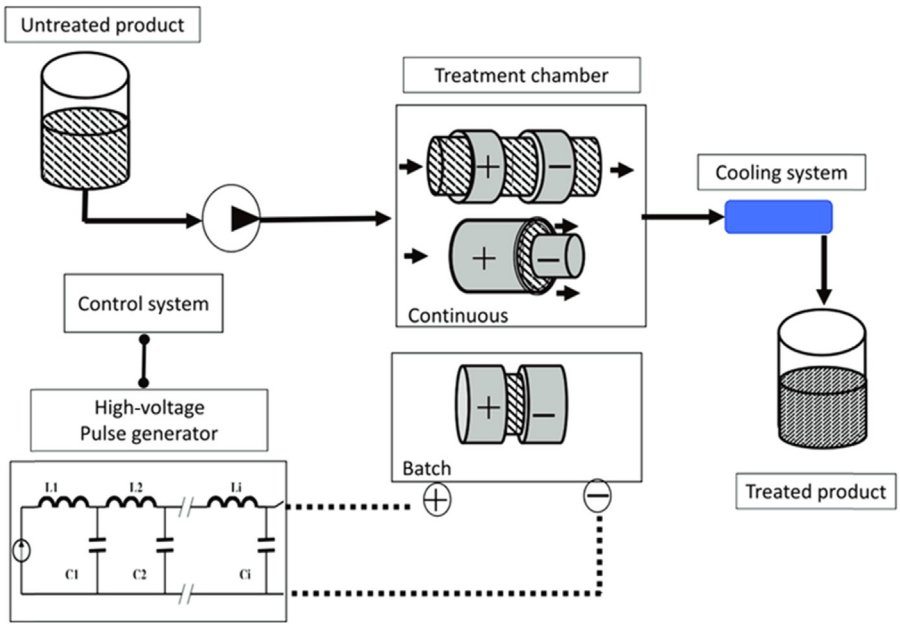

FIGURE 9.6 Use of pulsed electric field in yogurt manufacture.

characteristics such as mouth feel, shininess, clean cut, ropiness, and creaminess (Lin & Chien, 2007). In yogurt production, stabilizers may also be used to enhance the texture by improving firmness, minimizing whey separation (syneresis), and attempting to hold the fruit in the yogurt evenly mixed. Alginate (carrageenan), gelatin, gum (locust bean, guar), pectin, and starch are stabilizers used in yogurt.

9.6 Conclusion

Many technological processes such as centrifugation, homogenization, thermal processing, and in the case of yogurt, fermentation are used in milk processing and yogurt production. The consistency and sensory attributes of the finished product, whether it is milk or yogurt, are greatly influenced by each process and the conditions used in each process. Thermal processing involves pasteurization at low and high temperatures, treatment with ultra-heat and sterilization. The taste, microbial count, and the properties of milk proteins are affected by the implementation of heat processing in milk. UHP, US, microfluidization, and PEFs are other technologies that induce substantial changes in milk and yogurt made from treated milk. Therefore novel manufacturing treatment as an option may be suggested to maintain its nutrient benefit.

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Chapter 10

Pathogenic microorganisms in milk: their source, hazardous role and identification

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10.1 Introduction

Milk is recognized as an emulsion of lipid (triglyceride) globules in water-based fluid (containing aggregates of dissolved sugars and protein with minerals) (Rolf, 2007). Newborn mammals are nourished by the milk secreted through the mammary gland of the female (Van Winckel et al., 2011). It contains nearly all the nutrients required for the growth of infants, like source of energy (through lipids, lactose, and proteins), the raw material for the biosynthesis of nonessential amino acids, vitamins, inorganic elements, and essential fatty acids (Fox, 1997). Colostrum, present in the early lactation milk, carries the mother's antibodies for the infant to protect the newborn from a list of diseases.

Milk is an essential part of meals in various regions of the world because of its potential to be a good source of energy, protein, fat, and calcium. Milk is considered a nearly perfect food. Factors that vary among the milk of different species are type and proportion of protein, level of fat, sugar, vitamins, and minerals, the strength of curd, and size of butterfat globules (Rolf, 2007). If we look at North American and European diets, bovine milk provides dietary energy (about 8%), dietary fat (about 12%), and dietary protein (about 16%) (Muehlhoff et al., 2013). More than 50% of fat is contained in the milk of seals and whales, while the lowest fat content is observed in donkey and horse milk. Milk is composed of a list of properties that enhance the absorption and bioavailability of its nutrients (Wijesinha-Bettoni & Burlingame, 2013). Milk component like lactose helps in the absorption and utilization of micronutrients, like calcium, magnesium, phosphorus, and vitamin D in the intestine (Campbell & Marshall, 1975; Miller, 1989). In the acidic environment of the stomach casein micelles coagulate, which slows digestion, hence providing more time for the efficient digestion of milk nutrients (Lambers et al., 2013).

10.1.1 Production of milk around the world

The use of milk by humans was started in ancient times. The earliest traces of milk consumption from domestic animals were seen in people of North-western Anatolia (Asian portion of Turkey) in the 7th millennium BCE (Evershed et al., 2008). All around the world, milk is produced and consumed but the amount of production and consumption varies in different countries. Side by side, the per capita availability of milk is also different between countries. Generally, people around the world consume the milk of cows, water buffalo, goat, horse, camel, sheep, reindeer, and yaks. The main factors determining the presence of dairy animals are climate, feed, and water. Assumptions are also made that all around the globe a high number (nearly 150 million) of households spend their entire lives in milk production. Considering the factors influencing milk production, the socioeconomic structures of countries, markets, and dietary traditions play a crucial role in milk production in different parts of the world. Traditions related to the necessity of milk production are also seen in some countries, and in these countries milk and its product play an important role in the diet. In most of the developing nations, milk production is made by small holders as a source of livelihood, nutrition, food security, and cash income. Including this, in the developing nations the increased milk production is due to increase in the number of milk producing cattle rather than increase of per cattle productivity. In many developing nations, milk productivity is

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TABLE 10.1 Details of cattle strength, milk production, and consumption in leading milk producing countries (FAO, 2020).

Sr. no.	Region	Country	Milk production details
1.	South Asia	India	Milk production: 196.18 million tonnes Cattle strength: 192.5 million Milk consumption: 112 per kg per year
2.		Pakistan	Milk production: 47.30 million tonnes Cattle strength: 22.42 million Milk consumption: 9–12 per kg per year
3.	North America	United States of America	Milk production: 99.16 million tonnes Cattle strength: 94.8 million Milk consumption: 48.8 per kg per year
4.	EU	Germany	Milk production: 31.10 million tonnes Cattle strength: 12.9 million Milk consumption: 24.42 per kg per year
5.		France	Milk production: 25.01million tonnes Cattle strength: 20 million Milk consumption: 67 per kg per year
6.	Latin America	Brazil	Milk production: 35.17 million tonnes Cattle strength: 211.76 million Milk consumption: 57.6 per kg per year
7.	East Asia	China	Milk production: 32.67 million tonnes Cattle strength: 96.85 million Milk consumption: 36 per kg per year
8.	CIS	Russia	Milk production: 31.16 million tonnes Cattle strength: 19.8 million Milk consumption: 325 per kg per year
9.	South Pacific Ocean	New Zealand	Milk production: 21.79 million tonnes Cattle strength: 10.1 million Milk consumption: 105.26 per kg per year
10.	Middle east	Turkey	Milk production: 21.53 million tonnes Cattle strength: 16 million Milk consumption: 25 per kg per year

constrained due to poor quality feed, the low genetic potential of the animal to produce milk, and limited market and health services accessibility. A total increase of 59% has been observed in milk production in the past three decades. In the developing nations, South Asia has been playing a key role in milk production growth, while in Africa, growth of milk production is low due to adverse climate and poverty. There are some regions which are surplus in milk products such as Australia, France, Germany, US, and New Zealand. According to 2019 data, India was the largest producer of milk and its production is about 22% of the global milk. India's milk production is followed by the US, China, Pakistan, and Brazil (Table 10.1 FAO, 2020). In a study conducted by Food and Agriculture Organization (FAO), Israel dairy farms were found most productive milk farms. The milk yield depends mainly on three factors; production system, nutrition to the animal, and genetic potential of the animal. For example; Israel cows (with the highest yield) ate energy-rich mixed feed in barns; whereas, New Zealand cows (lowest yield) grazed all year (Table 10.1).

10.1.2 Processing of the milk

The processing of milk and its derived products is one of the crucial factors in the dairy industry field. The milk has a short shelf life and is highly perishable, and due to the presence of nutrients, it is a good medium for the multiplication of microorganisms, mainly pathogenic bacteria. The overgrown microorganisms are responsible for spoilage of milk and diseases in the consumers. Thus, careful handling of milk is required for its preservation, storage, and reduction in milk-borne illness. Processing of milk increases its lifetime, which is essential for milk storage, transport, and delivery.

The common approaches to remove microbes from milk are pasteurization, filtration, and ultra-high temperature treatment.

10.1.2.1 Pasteurization

Pasteurization is a very commonly used process of heat treatment of milk in the dairy industry (Milk pasteurization, 2017). This process kills the harmful pathogenic bacteria naturally found in raw milk and increases its shelf life. Though it is also the cause of loss of a few vitamins and minerals. The duration and amount of heat in pasteurization is based on the thermal death time of the target microbial population. In a broad term, pasteurization is grouped into two processes: low temperature or high temperature. The low-temperature pasteurization process enables the killing of all pathogenic microbes, and the high-temperature pasteurization process causes the killing of vegetative pathogenic and spoilage bacteria (Milk pasteurization, 2017). It also causes denaturation of serum proteins. Low-temperature pasteurization includes heating of milk to a high temperature (63°C for 30 minutes duration or 72°C for 15 seconds duration) and then immediate cooling (less than 3°C) (Olin, 1943; Ranieri et al., 2009). This process kills all pathogenic bacteria and reduces the load of spoilage bacteria. Including this, low-temperature treatment maintains the most physiological characteristics of milk. Pasteurization at 72°C for 15 seconds is also called high-temperature short time pasteurization or flash pasteurization. The high-temperature pasteurization process includes the heating of the milk to high temperature (138°C for 2 seconds, 89°C for 1 seconds and 90°C for 0.5 seconds) (IDFA, Pasteurization, 2020). Intense heating causes denaturation of serum proteins to control syneresis. The shelf life of milk can be increased till 9 months by using ultra-high temperature (UHT) pasteurization. In this treatment, the temperature of the milk is raised 135°C−140°C for 2−4 seconds. This process also targets *Coxiella burnetii* which is responsible for Q-fever. The following table may be used to understand the process of different types of pasteurization (Table 10.2).

10.1.2.2 Filtration

Filtration is also one of the popular techniques used in milk processing. Four different types of membrane filtration processes are used in the dairy industry such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. The filtration causes the removal of solid particles from the milk. Out of these methods, microfiltration causes the separation of bacteria, spores, and fat globules from the milk. Hydrophilic polyvinylidene fluoride microfiltration membranes have been used for milk sterilization, which enables separation of somatic cells, spores, bacteria, and yeast, while other components of milk were passed through it and retained in the milk. In this process, microbes are removed from milk, but the taste and nutrients of original milk are retained (Hui et al., 2004; Madaeni et al., 2011). Nowadays, filtration has revolutionized the dairy industry. The different types of filters are used to enhance the shelf life of the milk without heat treatment (Kumar et al., 2013).

Processing of the milk is done to convert milk into concentrated products which have high value, long shelf life, and are easily transported. Creaming and homogenization are also used in milk product processing. The common milk products are ghee, curd, and butter. The processing of milk has social importance in job generation as it provides off-farm job opportunities in the collection, transport, processing, packaging, and marketing of the milk products.

TABLE 10.2 Different schemes of pasteurization (IDFA, Pasteurization, 2020).

Sr. no.	Types of pasteurization	Pasteurization conditions
1.	Vat Pasteurization	30 min at 63°C
2.	High temperature for short duration	15 s at 72°C
3.	Ultra pasteurization (very high temperature for short duration)	2 s at 138°C
4.	Higher-temperature for short duration	1 s at 89°C
		0.5 s at 90°C
		0.1 s at 94°C
		0.01 s at 100°C

10.2 Microorganisms present in the milk and their sources

Milk is considered a complete food not only to humans but also to microorganisms due to its nearly neutral pH, high water content, and complex biochemical composition. Milk has high nutritional content and supports a rich diversity of microbiota (Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013).

The presence of pathogenic microbes in milk is a matter of concern for public health. The milk may spread many diseases to humans such as brucellosis, diphtheria, gastroenteritis, Q-fever, scarlet fever, and tuberculosis. The types of pathogenic bacteria present in the milk are dependent on the product and method of production and processing. Thus, proper milk handling and milk processing techniques have played important roles in minimizing the spread of milk-borne diseases. The source of these microbes is considered to be either endogenous or exogenous. Different microorganisms are associated with milk and have different possible sources (Table 10.3). The pathogens in milk may originate from the milking animals (cows) suffering infections or systematic diseases (Hunt et al., 2012), from machines, raw milk tankers, or associated personnel (Teh et al., 2011).

Microbes with endogenous origin are said to come directly from the cattle; whereas, those with exogenous origin comes from the external environment, like milking machines, and hands of the workers, etc. Milking is done by manual method, milking the cattle with their own hands in houses and small scale units and maintaining all the possible hygiene, and machine-based milking large scale dairy farms. Including the milking process, milk is collected, transported, processed, and delivered to consumers. This complete process gives a wide window to the pathogenic microbes (either coming from the external environment or from the ruminant itself) to contaminate the milk (Table 10.3).

The emissivity of bacterial transport through the entero-mammary pathway (i.e., leaving the intestinal lumen and reach the mammary gland through mesenteric lymph nodes), is influenced and conditioned by the physiological and hormonal changes that take place at the time of lactation and late pregnancy (Perez et al., 2007; Young et al., 2015). It is believed to be nature's way to stimulate the offspring's immune system to identify the molecular patterns which are associated with commensal microbes. But during milk processing, these microbes grow in sufficiently large number that they act as contaminants for the receiver.

It is also important to note that the udder skin and teat canal are not only responsible for the milk contamination. Direct contact with infected herds or contact with an ill environment (e.g., water, human) is also the cause of contamination (Brisabois et al., 1997). Silage is an effective source of milk contamination with spores (Te Giffel et al., 2002). Other sources for milk pathogens with exogenous origin can be mastitis, unhygienic milk practices, and the presence of environmental pathogens due to lack of animal hygiene (Oliver et al., 2009). Udder contamination can be the result of the contaminated production environment, soil and mud, feces, slurry, etc. Other microbial contaminations in milk may

TABLE 10.3 Different types of microorganisms associated with milk and their sources.

Source of microorganisms	Microorganisms
Endogenous origin	
Inside udder	*Streptococcus, Corynebacterium, Micrococcus*
Outside udder and teats	*Micrococcus, Staphylococcus, Enterococcus, Bacillus*
Exogenous origin	
Feces	*Escherichia coli, Staphylococcus, Listeria, Mycobacterium, Salmonella*
Milking machine	*Micrococcus, Streptococci, Bacillus, Coliforms*
Feed	*Clostridium, Listeria, Bacillus*, lactic acid bacteria
Bedding	*Clostridium, Bacillus, Klebsiella*
Feed	*Clostridium, Listeria, Bacillus*, lactic acid bacteria
Soil	Yeasts and molds, *Mycobacterium Bacillus Pseudomonas, Clostridium*
Water	*Coliforms, Pseudomonas, Coryneform, Alcaligenes*
Air	*Bacillus, Coryneform, Streptococcus, Micrococci*, yeasts and molds
Human	*Coliforms, Salmonella, Enterococcus, Staphylococcus*

be caused by long-duration storage under insufficient low temperature, inadequate milk pasteurization, contamination from bulk storage tanks, pipelines, equipment, and individuals associated with milking (Elmoslemany et al., 2009; Jørgensen et al., 2005; Kagkli et al., 2007; Lin et al., 2016). Milking machines are also said to be a big reservoir of microorganisms whose high population can be seen in the collected milk. It has also been observed that outside or inside feeding of the cow also influences the microbial community in the milk. Outdoor feeding milk has high *Staphylococcus* spp. (Hagi et al., 2010). The variety of microorganisms reported in milk were also traced in the farm, air, dust, hay, teat surface, and milking parlors. Other microbes, which were not from the farm environment, are said to be technologically related microbes like *Enterococcus, Lactobacillus*, and *Lactococcus*, as well as *Deinococcus, Leucobacter*, and *Paracoccus* (Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013).

Pasteurization has been established as an important procedure for the safety and consumption of milk and milk products, but these are some microorganisms in milk that are not completely inactivated during pasteurization and have ability to survive even after ultra-high temperature treatment. Thermoduric and spores-forming bacteria may survive in high temperatures. The Food and Drug Administration has declared the psychrotrophic, thermophilic, thermoduric, and spore-forming bacteria associated with the utmost risk of spoilage of dairy products (Hull et al., 1992). Out of these microorganisms, psychrotrophs have the capacity to survive at low temperature while thermophilic and thermoduric survive in high temperature.

10.3 Different types of microorganisms present in milk

The microorganisms found in milk can be classified in groups like pathogenic microbes (Table 10.4) and spoilage microbes. Further, they can also be classified based on their natural qualities such as psychrotrophic, spore-forming, thermoduric, etc. Spoilage microorganisms are those which deteriorate the texture, color, odor, or flavor of milk. This deterioration is possibly caused by microbial degradation of nutrients (sugars, lipids, and protein) present in milk. Psychrotrophs and thermoduric microbes involve *Bacillus, Clostridium, Pseudomonas, Corynebacterium, Arthrobacter, Lactobacillus, Mycobacterium, Micrococcus, Streptococcus*, etc. Psychrotrophs are mesophilic microbes that can grow at refrigeration temperature (though their optimum multiplication temperature is higher). Therefore, rapid cooling and upholding the low temperature of milk for a longer period (for storage and transport purpose), stimulates the growth of psychrotrophs (Martins et al., 2006). They are able to produce heat-resistant, extracellular or intracellular enzymes like pro proteases, lipases, and phospholipases which are responsible for milk deterioration (Samarzija et al., 2012).

Pasteurization is considered effective in reducing the microbial count of milk, but some bacteria survive pasteurization and these bacteria are termed as thermoduric bacteria. These bacteria are found associated with some contamination source; bacteria which are naturally present on skin, teats, and most of the mastitis-causing bacteria are not considered as thermoduric bacteria. Laboratory pasteurization count process is performed to detect the number of these bacteria in milk. This process also indicates the efficiency of hygiene measures and farm sanitation practices. Many strains of thermoduric bacteria have been isolated from processed milk products. Some of these are; strains of *Arthrobacter, Bacillus* (commonly isolated), *Clostridium, Enterococcus, Streptococcus* and *Lactobacillus*. (Cornell University, Dairy food science notes, 2007). *Bacillus cereus* and *B. circulans* are thermoduric bacteria that may grow under refrigeration. Thermophilic bacteria grow optimally at 55°C and up to 65°C in milk and cause contamination of milk products. These bacteria are generally spore-forming due to their ability to survive heat treatment. These bacteria grow as biofilm at suitable temperatures and are released into milk products. A common example of these bacteria is spore-forming bacilli and lactic acid bacteria (*Lactobacillus delbrueckii* and *Streptococcus thermophilus*) (Delgado et al., 2013; Seale et al., 2015).

Pathogenic microbes are those which cause milk-borne diseases, infection, or intoxication in a susceptible host due to the ingestion of contaminated milk. The consumption of raw and untreated contaminated milk having pathogenic microbes may be associated with zoonotic infections (van den Brom et al., 2020). These may cause serious health hazards to young children, pregnant women, elder people, and immunocompromised persons. These pathogens may affect the health of individuals severely. Typical symptoms for the diseases caused by milk-borne pathogens are fever, vomiting, diarrhea, nausea, and abdominal pains. In severe cases, infections may cause the death of an individual (Langer et al., 2012). Pathogenic microbes, generally causing milk-borne infections are *B. cereus, Listeria monocytogenes, Yersinia enterocolitica, Salmonella* spp., *Escherichia coli, Campylobacter jejuni, Brucella* spp., *Shigella* spp., *Staphylococcus aureus, Mycobacterium avium* ssp. *paratuberculosis* and mycotoxin producing fungi, etc. (Langer et al., 2012; Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013; van den Brom et al., 2020). It is also found that molds, especially *Aspergillus*, *Fusarium*, and *Penicillium* may produce mycotoxins in milk. These microorganisms

TABLE 10.4 Different types of microorganisms (bacteria, viruses, fungi) present in milk and diseases caused by these microbes.

Microorganism	Disease and symptoms	References
Brucella	Brucellosis (Undulant fever)	Jansen et al. (2019), Lindahl-Rajala et al. (2017)
Campylobacter jejuni	Gastroenteritis, Diarrheal disease; abdominal pain, fever etc	Jaakkonen et al. (2020), Schildt et al. (2006), World Health organization, Campylobacter (2020)
Coxiella burnetii	Q fever; chills, fever, weakness, headache, possible endocarditis	CDC. Q fever, (2019), Abdali et al. (2018)
Listeria monocytogenes	Listeriosis; flu like symptoms, miscarriage, stillbirths etc.	CDC (2020), Rosenow and Marth (1987)
Mycobacterium avium	Crohn's disease	McNees et al. (2015), Patel and Shah (2011)
Mycobacterium tuberculosis	Tuberculosis	Boukary et al. (2012), World Health Organization, Tuberculosis (2020)
Salmonella	Gastroenteritis, Typhoid fever; Diarrhea, nausea and high fever	CDC. Salmonella (2020), Olsen et al. (2004)
Staphylococcus aureus	produces toxin responsible for explosive vomiting, heat-stable enterotoxin, food poisoning	CDC. Staphylococcal (staph) food poisoning, (2018), Hagi et al. (2010), McMillan et al. (2016)
Yersinia Enterocolitis	Gastroenteritis; diarrhea, appendicitis	CDC. Yersinia enterocolitica Yersiniosis, (2016), Jayarao et al. (2006)
Escherichia coli	Gastroenteritis, hemolytic uremic syndrome (hamburger disease)	Lim et al., (2010)
Bacillus spp.	Diarrhea	(Bottone, 2010)
Tick-borne encephalitis viruses	Tick-borne encephalitis	Cisak et al. (2010)
Hepatitis A virus	Hepatitis A	Bidawid et al. (2000)
Nocardia asteroides	Bovine mastitis, fungal infection in human	Cook and Holliman (2004), Dhanashekar et al. (2012)
Candida krusei	Bovine mastitis, fungal infections in human	Şeker (2010)
Taenia spp.	Taeniasis	McFadden et al. (2011)
Toxoplasma gondii	Toxoplasmosis	Camossi et al. (2011)

contaminate milk through different routes. *S. aureus* contaminates the milk via teat canal, equipment, human handling, and by the environment. *C. burnetii* is shed through urine, feces, and milk. *Mycobacterium bovis* spread to humans by the consumption of contaminated raw milk. *M. avium* ssp. *paratuberculosis* (MAP) sheds through animal feces or milk into the external environment. It is also found that MAP is associated with Crohn's disease in humans (McNees et al., 2015; Patel & Shah, 2011). Shiga toxin-producing *E. coli* (STEC) sheds from an animal though feces and contaminates the milk. *Y. enterocolitica* is shed into feces from animal and cause acute gastroenteritis (Schiemann & Toma, 1978). *L. monocytogenes* is directly excreted into milk and causes listeriosis (Hunt et al., 2012). *Brucella* spp. is excreted from the animal into milk and cause brucellosis in humans. *Brucella* and *Listeria* are supposed to survive during pasteurization. Mycotoxins (aflatoxin, fumonisins, ochratoxin, trichothecenes, zearalenone, etc.) are secreted by filamentous fungi (*Aspergillus, Alternaria, Fusarium*, and *Penicillium*) as secondary metabolites and cause headache, nausea, diarrhea, and cancer (Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013). Some microorganisms present in milk and milk products are discussed below.

10.3.1 Bacillus

Bacillus usually form heat-stable spores and survive pasteurization, they produce rennet like enzymes which tend to coagulate milk. Based on suitable temperature required for their growth, these strains may be the type of psychrotrophic, mesophilic, or thermophilic. Milk casein and polysaccharides are hydrolyzed by the enzymes produced by *Bacillus subtilis*. Species of *Bacillus* such as *Bacillus stearothermophilus* and *Bacillus macerans* are good at survival in milk cans, resulting in spoilage and sweet curdling defect. A very little amount of gas formation may be observed in this process (Durak et al., 2006; Ombui & Nduhiu, 2005).

10.3.2 Clostridium tyrobutyricum

Clostridium tyrobutyricum is a thermoduric, anaerobic, and spore-forming bacteria responsible for late gas defect in cheese (late blowing in cheese) resulting in its high pH, high moisture content, and low interior salt content (Klijn et al., 1995; López-Enríquez et al., 2007). *C. tyrobutyricum* is reported to produce gases (carbon dioxide and hydrogen), and acids (butyric acetic and acetic acids) during fermentation (Liu et al., 2006).

10.3.3 Pseudomonas

Pseudomonas produce heat-stable lipases, hence degrade fats and produce lipolytic rancidity. One of the most commonly isolated species from milk is *Pseudomonas fluorescens*. *Pseudomonas putrefaciens* causes spoilage by forming taint in butter (Bryan, 1983; Dogan & Boor, 2003; Scheldeman et al., 2005).

10.3.4 *Coryneform* bacteria

Coryneform bacteria are gram-positive rod-shaped and nonspore-forming bacteria. These bacteria are found on surface-ripened cheese. Cheese smear *Coryneform* are generally psychrotrophic in nature and unable to show growth at 37°C, while pathogenic *Coryneform* bacteria are found facultative anaerobes, and these bacteria show growth at 37°C. This cheese smear group contains bacteria such as *Arthrobacter, Brevibacterium, Corynebacterium, Microbacterium* and *Rhodococcus*. These bacteria have the capability to survive in high salt concentrations and synthesize many types of proteolytic enzymes. Thus, these microorganisms cause slimy rind, discolored appearance in cheese, and undesirable flavors (Hassan & Frank, 2011).

10.3.5 Lactobacilli

Lactobacilli are facultatively anaerobic, rod-shaped and gram-positive lactic acid bacteria which are responsible for producing numerous proteolytic enzymes. *Lactobacillus casei* and *Lactobacillus plantarum* causes the ripening of the cheddar cheese. Due to excess gas formation, these microbes cause open texture in cheeses. *Lactobacilli* causes the conversion of L (+)-lactate into D(−)-lactate which involves in the formation of calcium lactate crystals from calcium (Kagkli et al., 2007; Stiles & Holzapfel, 1997). The calcium lactate crystals appear as white spots on the surface of the cheese. Though calcium lactate is not harmful, it is considered as a quality defect (Swearingen et al., 2004).

10.3.6 Micrococcus

Micrococcus species are capable of surviving during the process of pasteurization, and they have the tendency to produce swelling in UHT milk packs. The presence of *Micrococcus* in cheese may either beneficial or unfavorable, depending on the types of cheese and type of *Micrococcus* sp. (Hassan & Frank, 2011).

10.3.7 Coliforms

Coliforms are gram-negative, nonspore-forming rod-shaped microbes. As they are nonspore forming, they cannot withstand pasteurization temperatures. The most common genera included in coliforms are *Citrobacter, Escherichia, Enterobacter, Klebsiella, Proteus*, and *Serratia*. These microbes commonly cause spoilage in cheese along with the formation of acid and slime in cottage cheese, bitter flavors, and formation of grassy, unclean, medicinal, or fecal odors in cheese (Jayarao & Wang, 1999; Khayat et al., 1988; Van Kessel et al., 2004).

10.3.8 Listeria monocytogenes

L. monocytogenes are gram-positive, rod-shaped with coccoid or diphtheroid morphology and nonspore-forming bacteria, which is responsible for stillbirths or deaths of infants soon after birth. Major problems caused by this microbe in humans include meningitis, infectious abortion, perinatal septicemia, and encephalitis (Rosenow & Marth, 1987).

10.3.9 Yersinia enterocolitica

Y. enterocolitica is nonspore-forming, rod-shaped gram-negative bacteria. *Y. enterocolitica* can be destroyed by pasteurization, but if contamination occurs after pasteurization, then it can grow at refrigerator temperature. Illness caused by this microorganism shows symptoms like fever, abdominal pain, and diarrhea (Jayarao et al., 2006).

10.3.10 Salmonella

Salmonella species are nonspore-forming, hence, can be destroyed by pasteurization. Its common pathogenic strains found in milk are the serotypes *Enteriditis* and *Typhimurium*. It causes salmonellosis, whose symptoms are diarrhea, fever, and abdominal cramps (Nero et al., 2008).

10.3.11 Escherichia coli

E. coli is rod-shaped, gram-negative, and nonspore-forming bacteria. The four groups of this microorganism have been recognized in milk as; enteropathogenic, enterotoxigenic, enteroinvasive, and colehemorrhagic (Kornacki & Marth, 1982; Vasavada, 1988). Diseases like hemorrhagic colitis (HC) or bloody diarrhea are caused by *E. coli* (Lim et al., 2010). At higher risk, its contamination causes hemolytic uremic syndrome (HUS), which is characterized by serious kidney dysfunction causing the blood urea (Van Kessel et al., 2004). Another, pathogen concerned with the milk is STEC. STEC is also called verotoxin-producing *E. coli* (Januszkiewicz & Rastawicki, 2016). The main reservoir of STEC is recognized to be cows, but it is also present in other domestic animals like sheep and goats, and shed through their feces without showing any symptoms. In an improper hygiene environment, milk may be contaminated at the time of milking or processing of milk. Researchers have identified nine virulence genes associated with STEC strains. Out of these genes, *stx1* and *stx2* are found associated with strains of bovine milk, while genes *stx1c* and *stx2b* are commonly found associated with strains present in goat and sheep milk (Martin & Beutin, 2011).

10.3.12 Campylobacter jejuni

C. jejuni is a gram-negative and nonspore-forming bacteria. These bacteria exhibit characteristic morphology such as S, gull, or comma-shaped. Raw milk is considered a common source of *C. jejuni*. *Campylobacters* are one of the leading microorganisms associated with zoonotic infections and cause campylobacteriosis (gastrointestinal problem). It requires fastidious conditions, and thus is difficult to isolate from milk. Symptoms shown by its illness are mild enteritis, sometimes severe enterocolitis, followed by abdominal cramps, nausea, and bloody diarrhea (Doyle, 1981; Modi et al., 2015).

10.3.13 Virus

A series of viruses may also exist in milk-borne infections. Some viruses require slightly higher-heat inactivation temperature than maintained in pasteurization but in some countries, postpasteurization viral contamination is also observed. Some milk contaminating viruses are tick-borne encephalitis viruses, hepatitis A and hepatitis E virus (Bidawid et al., 2000; Cisak et al., 2010; Dhanashekar et al., 2012).

10.3.14 Fungi

A range of pathogenic fungi infecting the udder of the cow may be excreted from the cow in milk. Fungal species causing bovine mastitis such as *Nocardia asteroides, Nocardia brasiliensis, Candida albicans, Candid tropicalis*, or *Candida krusei* have been found excreted in milk and may be transmitted to humans through improperly treated milk (Cook & Holliman, 2004). The contaminated milk may be a source of fungal infection mainly in immunocompromised

patients such as diabetic, HIV-positive with decreased CD4 count, and patient with cirrhosis. (Dhanashekar et al., 2012).

10.3.15 Parasites

The different types of parasites such as *Taenia* sp., *Toxoplasma gondii, Ascaris lumbricoides*, and *Trichuris trichiura* have been found in contaminated milk, and these parasites may be transmitted to humans from contaminated milk. The sources of infection of these agents into milk are from the environments of milk procurement and soil (Camossi et al., 2011; Dhanashekar et al., 2012; McFadden et al., 2011) (Table 10.4).

10.4 The economic significance of pathogenic microbes

The dairy market is generally considered as one of the highly regulated agricultural markets. Dairy-related activities also contribute to many nonmarket-based economic benefits such as manure to be used as fuel or organic fertilizer. Milk production activity provides regular income to producers. In developing countries, milk production provides daily and regular income to a large number of families in rural areas. The price of milk at the farm gate is calculated by many factors such as composition and cleanliness. However, in developing countries, the price of milk is commonly based on the fat content of the milk. But it is not helpful all the time because sometimes cleanliness of milk is ignored, as cattle get severe diseases, too, which not only alter the economy of one's dairy but also of the country if the disease spreads to other dairies.

The economic consequences due to diseased cattle are related to disease treatment, production losses, culling, and changes in milk quality. One of the major diseases encountered in dairy is mastitis, which causes about 3%−5% milk loss of milk yield production (Oliver & Calvinho, 1995). The change in milk composition and productivity is affected by the level of infection and duration of infection in the mammary gland and the presence of somatic cells. The high number of somatic cells counts in milk suggests an incipient mammary gland inflammatory response (Damm et al., 2017; Frössling et al., 2017). It is found that subclinical mastitis causes 10%−26% of total milk loss (DeGraves & Fetrow, 1993). Including these losses, there are additional losses caused by variation in the composition of milk, which create interference in milk manufacturing processes, increased treatment cost, and culling.

10.5 Control of contamination of milk by microorganisms

There are methods to control pathogenic contamination in milk as well as for the treatment of diseases. The pre and postmilking sterilization processes significantly decreases the infection in milk, while udder hygiene controls mastitis and cow pathogen transmission (Oliver et al., 1999). Nevertheless, modern milk processing practices emphasize herd inspections, sanitation, improved udder health, proper handling and storage of raw milk, and almost universal pasteurization, thereby minimizing the potential threat of pathogenic bacteria and the number of outbreaks (Bryan, 1983). Researchers also advise the use of certain chemicals as sanitizers such as those containing chlorine and iodine, acid anionic compounds, and quaternary ammonium-based sanitizers; they have been researched to be effective against *Salmonella typbimuriurn* and *L. monocytogenes* (Lopes, 1986). In the case of dairy manufacturing plants, special focus is given on the maintenance of instruments, the use of footbaths, the use of clarifiers, and control of the environment of the plant (Surak & Barefoot, 1987). The most common method used for decontamination of milk is pasteurization; it kills most of the pathogens but not the spores. Also, milk is usually pasteurized before packaging; therefore, postpasteurization contamination can occur.

10.6 Identification methods of milk-borne pathogens

Improper processing of milk samples may cause transmission hazards to many pathogens and cause outbreak of brucellosis, listeriosis, and tuberculosis, etc., which poses a threat to many countries (Dhanashekar et al., 2012). After taking all the major steps to avoid pathogenic contamination in milk, it has to undergo a list of tests to ensure that the milk delivered to our home is really contamination free. Various biochemical tests and molecular analysis tests are done to fulfill this purpose. Tests are designed in such a way so that the pathogens can be detected in their early stages of growth, which could reduce the number of dairy borne outbreaks. Under the process of testing and analysis, the first and the most important step is sampling. The sampling point varies from industry to industry. Most of the sampling points are milk tanker wash, storage silo; swabs from the milk packing rolls, crates using storage, and from the workers

involved in packaging, random milk packs; butter sample from continuous butter-making machines and sample of butter wash, swabs from butter handlers, plastic sheets used in butter packing; random sampling of ice cream, cheese, sweets buttermilk, flavored milk, and curd; swabs from the handlers in respective units; water samples from wash water and effluent treatment plant (Dhanashekar et al., 2012).

Separate categories of methods are developed for the analysis of the food/dairy pathogens like microbiological, microscopy, sensory, physical, and physio-chemical. Common methods used for the analysis of milk samples are discussed below

10.6.1 Phenotypic methods

Phenotypic methods involve the culturing of microorganisms in culture medium followed by morphological, biochemical, or physiological characterization (De Boer & Beumer, 1999; Quigley et al., 2011). These methods are still used to determine the quality of milk. Milk microbes, which are frequently tested via phenotypic method, include thermoduric populations (which resist pasteurization), coagulase-positive *Staphylococci*, sulfate-reducing *Clostridia, B. cereus, L. monocytogenes, Enterobacteriaceae, E. coli*, coliforms, and Salmonella (Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013). This approach is considered low-tech, labor-intensive, time-consuming, and lacks efficient characterization of each species of microorganism (Zhao et al., 2014). Biochemical tests of milk samples are performed to detect the presence of urea, detergents, formalin, salt and sugar, etc.

10.6.2 Standard plate count method

Different milk and dairy samples are analyzed to detect the presence of microorganisms. Generally, milk or milk products are tested for the presence of coliforms and yeast and molds; whereas, the standard plate count (SPC) method is commonly employed to find the presence of microorganisms (mainly coliforms) in raw milk (Dhanashekar et al., 2012). The SPC method is used to detect the presence of aerobic bacteria that require growth conditions as incubation temperature between 30°C and 35°C.

Selective culture media are used to differentiate and distinguish microorganisms present in the milk. As per the International Dairy Federation standards, the SPC is conducted using nonselective media (such as plate count agar), while the MacConkey agar culture medium is used as a selective medium for detection and isolation of gram-negative bacteria. The recommended culture conditions for the SPC are 30°C/3 days or 32°C/2 days. Single-strength lactose broth (4% w/v in distilled water) or double-strength lactose broth (8% w/V in distilled water) are used for the detection of gas-producing coliforms. To detect the presence of yeast and mold, 10% of tartaric acid is added to the culture medium to inhibit the growth of coliform and other spore-forming bacteria. Swabs are used to collect culture from the respective sites or suspected sources. The swabs are taken in small vials containing sodium chloride or trisodium nitrate solution. Petri plates, pipettes, test tubes, and other culture medium are sterilized. After inoculation, plates are incubated at 35°C for 24 hours in a biochemical oxygen demand (BOD) incubator. Different types of microorganisms require different durations for incubation such as coliform (24 hours), yeast (3 days), and molds (5 days). After the given time, the plates are examined for the presence of microbes. The presence of airborne pathogens is analyzed at a particular location by exposing the sterile culture medium plate having MacConkey agar to air for about 5 minutes and incubation in an incubator (Dhanashekar et al., 2012). Generally, different methods are used for microbial analysis of pasteurized and raw milk. For total bacterial standard methods, agar is used; for coliform, violet red bile agar is used; and somatic cells are examined by direct microscopy of blood (Milk Facts, 2020).

10.6.3 Molecular and genotypic methods

To avoid the drawbacks of phenotypic methods, people rely on rapid and high throughput strategies involving the detection of genetic makeup like DNA\RNA, and this approach is known as the genotypic method. Other than overcoming the disadvantages of conventional methods, DNA-based pathogen detection, assays have several advantages such as sensitivity, rapidity, and selectivity (Zhao et al., 2014). Molecular genetic techniques are found to be more discriminating than conventional methods and also provide the right taxonomic information about the strain for pathogenic surveillance (Henri et al., 2016). They can detect a very low number of organisms in the sample (Lee et al., 2015).

The rapid and reliable method for identifying pathogenic cells, especially bacterial, is polymerase chain reaction (PCR) amplification and its modification\s like qPCR, multiplex PCR etc., are used to confirm the results obtained by traditional methods. This technology is able to detect the presence of those microorganisms that do not isolate through traditional culture methods. Focus on DNA-binding agents or RNA eliminates the risk of false-positive results obtained

from the amplification of DNA of dead cells (Quigley et al., 2011). Through molecular analysis, it was revealed that refrigeration of milk samples decreases bacterial diversity (Raats et al., 2011).

This technique is used along with its variants known as PCR-denaturing gradient gel electrophoresis and PCR-temporal temperature gradient gel electrophoresis or single-stranded conformation polymorphism (He et al., 2009; Ndoye et al., 2011; O'Sullivan et al., 2013; Postollec et al., 2011; Quigley et al., 2011; Raats et al., 2011). It is also suggested that these methods may be employed in conjunction with DNA sequencing (Chiang et al., 2012; Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013). Therefore, oligonucleotide probes or highly conserved regions such as 6S or 23S rRNA genes can be selected to detect target specific genes.

Such methods lack the information about bacterial colony location and its distribution in the dairy food. For this reason, research is shifted towards nondestructive microscopic methods like confocal laser scanning microscopy, cryo-and regular scanning electron microscopy, and transmission electron microscopy (Hickey et al., 2015).

The molecular methods (PCR based) detect specific genes of pathogenic microbial strains from contaminated milk and its products (Hennekinne et al., 2012). PCR is widely used to detect pathogens like *E. coli, S. aureus, Salmonella*, and *L. monocytogenes* (Lee et al., 2015; Wang et al., 2015). Real-time quantitative PCR (qPCR) is more advanced than conventional PCR in terms of quantification, real-time, and in situ analyses (Rasolofo et al., 2010; Riyaz-Ul-Hassan et al., 2013). Here PCR products are detected via a fluorescent signal, as they accumulate during the process (Auvolat & Besse, 2016). Investigators have used a culture-dependent approach and direct molecular method in combination with cloned libraries of 16S rRNA gene and Q-PCR to get more detailed information of the composition and the dynamics of milk microflora (Rasolofo et al., 2010). Multiplex PCR detects as well amplify multiple target sequences in a single amplification reaction by using a different set of primers. In one study the clone library sequencing approach has been able to detect *Chloroflexi, Cyanobacteria, Planctomycetes, Verrucomicrobia*, and unclassified bacteria, at low levels (Verdier-Metz et al., 2012). However, some of the microorganisms (such as *Arcanobacterium, Clavibacter*, and *Solobacterium* sp.) which were identified on the teat surface were not detected in milk (Verdier-Metz et al., 2012).

One more effective method for samples containing a mixture of nucleic acids is DNA microarray. It works on the concept of hybridization, that is, binding of targets to the probes on the array plate. It allows the measurement of the relative concentration of the nucleic acids in the sample. Hence, the use of biochips in the food and dairy industry is increasing these days, for the concurrent detection and identification of multiple types of microorganisms in a short time period (Chiang et al., 2012).

Molecular subtyping methods are also been used for a decade for the investigation (surveillance and outbreak) of milk-borne diseases (Deng et al., 2016). Molecular subtyping used molecular techniques like whole-genome sequencing (WGS), pulsed-field gel electrophoresis (PFGE), and multiple-locus variable-number tandem repeat analysis (MLVA). PFGE is the most commonly used method among these, due to its reproducibility and high discriminatory ability (Deng et al., 2016). It was initially used for the detection of *E. coli*, but nowadays it has been developed for typing of other bacteria like *L. monocytogenes, Salmonella*, and *Vibrio parahaemolyticus* (Liu et al., 2016). PFGE is time-consuming and laborious, therefore, not suitable for typing several isolates. These limitations of PFGE were overcome by MLVA, which is a rapid method with highly reproducible results. MLVA technique uses amplification and size analysis of the number of repeats in the variable-number tandem repeats region present in the bacterial genome (Bertrand et al., 2015). Commonly typed bacteria via MLVA include *Salmonella, E. coli* and *V. parahaemolyticus*. The more recent and advanced technique used for typing is whole-genome sequencing, which applies next-generation sequencing. It is used for sequencing a large number of isolates, and including these methods, bioinformatics tools are also used for analyzing the phylogeny (Loman et al., 2012; Revez et al., 2014). It is used for various bacterial stain detection such as *E. coli, Campylobacter, Listeria* spp. and *Salmonella* spp. WGS is expensive and also requires adequate bioinformatics skills for genomic analysis (Bopp et al., 2016; Burall et al., 2016).

Each type of cheese contains its unique pool of microbial communities. High throughput sequencing techniques have been reported to facilitate the more precise detection of the diverse microbiome that existed in fermented cheeses, study, and characterization of the microbiome dynamics during the cheese ripening process, as well as the role of the microbial population in the formation of specific organoleptic and physio-chemical properties. This technique assists the cheese makers to evaluate the quality and safety of the product. For analysis of microbial communities, this technique uses three major approaches: amplicon sequencing, shotgun metagenome sequencing, and metatranscriptome sequencing (RNA-seq), (Kamilari et al., 2019).

In one research, investigators performed the study of microbial communities of raw and pasteurized milk through real-time quantitative PCR, flow cytometry, and high throughput sequencing. These techniques allowed the identification of diverse bacterial populations in cow milk and identified the bacteria genera for the first time. These genera were *Bacteroides, Catenibacterium, Faecalibacterium*, and *Prevotella*. These techniques also identified the diverse bacterial cultures in pasteurized milk and nonthermoduric bacteria in the damaged and nonculturable form (Quigley, McCarthy et al., 2013; Quigley, O'Sullivan et al., 2013).

Single-molecule, real-time sequencing technology (SMRT) has been reported to detect microbial population using full-length 16S rRNA gene (Amir et al., 2013; Zhang et al., 2015). This technique has been used to evaluate the milk samples (raw, UHT, and infant formula) for the presence of microorganisms (Hou et al., 2015).

10.6.4 Flow cytometry

The flow cytometry technique has been employed as a routine process to measure the presence of somatic cells in milk and to diagnose the udder health and milk quality (Li et al., 2015). Flow cytometry uses fluorescent stains for microbiological analysis such as Oregon Green conjugated wheat germ agglutinin (binds to the bacterial cell wall) and hexidium iodide (binds with bacterial DNA). This technique analyzes the microbial count of bulk milk samples within a short time. The analysis of milk samples is hampered by lipids and proteins, and thus enzymatic clearing of milk samples is conducted (Gunasekera et al., 2000). Flow cytometry had also been reported in the microbiological analysis of milk samples; it provided the diagnosis of the microbial population in milk samples into many categories such as bacteria mainly related to lack of hygiene, psychrotrophic hygiene, and bacteria mainly associated with mastitis (Holm et al., 2004). The study of thermophilic bacteria in the milk powders is also performed using this method (Flint et al., 2007).

10.7 Microbiological standards of milk

Microbial standards of the milk are used to describe the acceptable limit of microorganisms in milk. The dairy regulations vary in different regions and may have different standards. It is suggested that prepasteurized milk sample (for grade A use) should not have more than 100,000/mL total bacteria at the individual producer level and 300,000/mL total bacteria at the commingled level. While the presence of somatic cells is required to be below 750,000/mL, this milk should not show a positive test for drug residue. In the case of pasteurized milk (grade A) the total bacterial count is acceptable below 20,000/mL and coliform below 10/mL. As per the New York State regulations for raw milk, it is suggested that the maximum limit for the total bacterial count in raw milk is 30,000/mL and 750,000/mL for somatic cells. While for drug residue detection, no positive test is required (Milk facts. Microbial standards for milk, 2020).

10.8 Conclusion and future perspectives

Milk quality is the essential element to be considered at the level of the milk-producing and processing. The nutritive components of the milk make it a good medium for the growth of microorganisms. Milk contains complex microbial communities that include microorganisms that are not good for human health and the presence of such type of microbial community in milk is a concern with food quality perspective. Due to this, milk microbiota is the focus of continuous attention. There are many factors that influence the presence of microorganisms in milk such as the teat canal, the surface of teat skin, and feed. Including these factors, environmental factors, water quality, housing hygiene, and equipment hygiene also influence the presence of microorganisms in raw milk. The microbial communities in raw milk are also affected by the presence of thermoduric bacteria and other microorganisms associated with postpasteurization contamination (psychrotrophic bacteria like *Pseudomonas*). The presence of pathogenic microorganisms cause serious health hazards to humans and become a potent source of zoonotic infections. Thus, the consumption of milk contaminated with pathogenic microorganisms may represent a serious health risk to consumers. Due to the severity of many of the milk-borne diseases, the milk samples must be constantly accessed for the presence of pathogens. On a broad scale, the identification of microorganisms is performed by phenotypic (based on culture and biochemical test) and genotypic methods. The common traditional techniques used to detect and count bacterial contamination in milk use growth of bacteria on culture media. These methods are considered as labor-intensive, not easy to handle, time-consuming, and results may be confusing due to the variable microbial phenotypes. Including this, microorganisms present as subdominant populations, or which cannot be easily grown in the laboratory, remain undetected in this approach (Paszyńska-Wesołowska & Bartoszcze, 2009). Moreover, culture-independent techniques such as flow cytometry and molecular diagnosis (DNA-based methods) have provided significantly improved results of the bacterial analysis. DNA-based methods are rapid and specific. These methods include PCR, PFGE, real-time PCR, and microarray. But the molecular methods are not very commonly used and need to be implemented on a large scale.

The key objective of the milk or dairy industry is to maintain the production of milk according to demand and provide safe and healthy milk and milk products to consumers. Including this, the dairy industry is also associated with social and economic development. The sources of the microbial community are endogenous and exogenous; thus, detection of pathogens and the practices that may cause a reduction in exposure of microbes to milk are more crucial. It

becomes pertinent to maintain hygiene at the farm level at the time of milking, transport, and storage. The overall efforts must be focused on animal health and farm hygiene, equipment hygiene, personnel sanitation, hygiene at milk processing, and storage.

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Chapter 11

Fermented pastes using dairy important microbes

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11.1 Introduction

In most countries, each and every meal has some form of paste on the margins of the plate as an accompaniment. Every human's love for it remains a palate-pleasing thread that binds the country's multicultural eating practices together. This paste can natural or fermented, which is prepared and preserved using several methods from raw plant parts. The preparation and preservation of the traditional pastes made from various vegetables, pulses, and spices using indigenous functional microbial consortium ensures better food security (Galimberti et al., 2021). Foods can be prepared from traditional and industrial fermentation methods. Fermentation which was invented by our ancestors is a good way to preserve perishable raw materials and improve the nutritional value of normal food. The fermented paste is used as a condiment, generally as a side dish/dip/toppings for several food items. They not only work as a taste enhancer, but also are nutritious and improve the quality of food. These pastes are having versatile characteristics in the area of health functionality, physicochemical properties, and quality analysis produced by different processing methods. In our day-to-day life, the access to the diary-based fermentation (Pongsetkul et al., 2014), vegetable-based fermentation, soybean-based fermentation, and meat-based fermentation (Liu, 2011). Some of the pastes are fermented by probiotic microorganisms like lactic acid bacteria (LAB) and, therefore, they help in better digestion, absorption, and assimilation of nutrients. LAB are of major economic importance to the food industry as they are involved in fermentation processes that change the paste of different plant-based foods. These natural microflora dominate much of the fermented pastes, where they serve a preservative or a spoilage role. They also play an important role in the digestive tract of humans and animals, especially the young. LAB dominates the natural microflora of milk, meats, vegetables, and cereal products in an appropriate environment. The fermented paste is the result of fermentation triggered by microbes introduced from nature. An introduction of useful microbes for fermentation is desirable, whereas the introduction of harmful microbes would affect the quality of fermented foods. Fermentation of food is the technology that utilizes the growth and the metabolic activity of a microorganism for the transformation and stabilization of a food product. The basic idea for fermentation is to increase the perishability of the food product. The desirable bacteria increases the shelf life of food by inhibiting the growth of the pathogens or spoilage microorganisms. Some fermentation process lowers the pH of food products, hence, preventing harmful microorganisms to grow or survive by providing them harsh acidic nature. The control fermentation processes developed the growth of beneficial bacteria, which prevents the growth of bad microbes. Based on the type of food fermented or the type of fermentation, nonperishable food products can be stored for several years. Many studies have shown that these fermented pastes have the ability to inhibit cholesterol, lower blood pressure, remove radioactive substances, prevent gastric ulcer, and as an antitumor and antioxidant (Kim et al., 2012).

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11.2 Types of pastes

In various countries with diverse culture, the evolution of native plants and traditions developed various types of culinary fermented paste of varied nature (Table 11.1).

11.2.1 Fermented shrimp paste

Commonly known as "Kapi" by the people of Thailand, kapi is a fermented paste made of shrimp prepared from the planktonous shrimp or krill (*Acetes vulgaris* or *Mesopodopsis orientalis*). To make the paste, the ratio of salt:shrimp is 1:3. The color of a fermented shrimp paste varies. It may be a purple, pink, gray, or dark brown in color. It may have a soft or hard consistency. It could be prevented for spoiling for several months. However, variation in raw material, shrimp:salt ratio and fermentation time period can lead to difference in characteristics of the final product . Kapi is a high protein product as well as containing a high salt concentration.

11.2.2 Fermented soybean paste

Fermented soybean has its origin in Korea. It's one of the most essential and oldest condiments in Korea. Commonly known as Doenjang, it is made from soybeans and brine. Microorganisms involved in the fermentation process include *Bacillus subtilis* and *Aspergillus oryzae*. Final product appearance is a thick brown paste. The paste has a pungent smell similar to that of a ripened blue cheese. The paste can be consumed as a condiment in raw-paste form with different dishes, as flavored seasoning, or even as a dip. Soybean paste is rich in vitamins, secondary metabolites, minerals, and plant hormones (phytoestrogens), which are sometimes claimed to have anticarcinogenic properties (Kim et al., 2010).

11.2.3 Fermented red pepper paste

Fermented red pepper paste, commonly known as Gochujang by the people from Korea, has the following characteristics; it has a pungent smell, a spicy flavor, and a sweet taste (Lee et al., 2016). The product is known to be rich in different nutrients such as amino and fatty acids, organic acids, and different sugars. These are usually produced during the fermentation process as raw materials. Fermented red pepper is also known for its various health benefits; these include antitumor, cancer and obesity effects.

11.2.4 Fermented fish paste

It is the conversion of organic compounds into simple compounds like amino acids, peptides, and various nitrogenous compounds. It may be by the activities of microorganisms or endogenous enzymes. This procedure is known as fish fermentation. Fish paste is a popular condiment in different dishes. It is made by fermenting fish with salt 20−25% w/v under controlled conditions. The product has a high nutrition value, a unique flavor, and long lasting shelf life. Fish paste can be considered a protein source and PUFA (polyunsaturated fatty acids). However, these could be damaged by severe fermentation conditions. The product has an extremely pungent smell and a salty taste, usually thick and whitish in color.

11.2.5 Fermented black garlic paste

Heating whole garlic bulbs (*Allium sativum*) over a period of several weeks results in black cloves. The resulting cloves are used to make the fermented paste. The product has a sweet taste with tangy undertones to it. A good fermented black garlic paste is characterized by a longer aging period to bring out powerful antioxidants and will also have no odors associated with garlic.

11.2.6 Fermented milk tomato paste

The tomato (*Lycopersicon esculentum*) and its products contain lycopene, a carotenoid and an antioxidant, that plays an important role in the health of human beings (Erge & Karadeniz, 2011). Lycopene is present in even higher content in tomatoes made into a paste. Tomato components can be fermented by LAB such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus casei*. The paste has a bright red color, however color may vary depending on tomatoes used or fermentation time.

TABLE 11.1 Major characteristics of different fermented paste available Globally.

Sr. no.	Name of the fermented paste	Characteristics	Region	Country	References
1.	Cheonggukjang	A fermented soybean paste used in Korean cuisine. It contains a whole as well as ground soybean. prepared in 2−3 days through fermentation of boiled soybeans by adding *Bacillus subtilis*, which is usually contained in the air or in the rice straw, at about 40°C without adding salt	East Asia	Korea	Lee et al. (2013)
2.	Dajiang	This is a fermented paste produced entirely from soybean and brine. It is a by-product of soup soy sauce production. It is used as condiment in raw paste, flavored or dipping condiment	East Asia	China	Xei et al. (2019)
3.	Doubanjiang	Hot and savory Chinese bean paste made from fermented broad beans, chili peppers, soybeans, salt and flour	East Asia	China	Li et al. (2016)
4.	Gochujang	Traditional seasoning and condiments which is savory, sweet, and spicy popular in Korean cooking. It is made from *meju* (fermented soybean) powder, *yeotgireum* (barley powder) and salt	East Asia	Korea	Kim et al. (2017)
5.	Terasi	Terasi is an Indonesian shrimp paste that is traditionally fermented and is widely consumed by Indonesian people	Indonesia	Indonesia	Kobayashi et al. (2003)
6.	Huangjiang	This paste is made from yellow soybean, salt, and water. Wheat flour, though not formerly used, is often used as an additional ingredient in the modern-day, and potassium sorbate is used as a preservative	East Asia	China	https://en.wikipedia.org/wiki/Yellow_soybean_paste
7.	Kinema	The slimy, odorous product of fermentation is traditionally prepared into a soup of soybean that is consumed with rice but can also be turned into a savory dip or a pungent side dish to be consumed along with rice or bread. Kinema is considered a healthy food because fermentation breaks down complex proteins into easily digestible fractions	South Asia	Nepal, Northeast India	Tamang et al. (2015)
8.	Miso	Miso is Japanese seasoning produced by fermenting. miso is a traditional produced with salt and *kōji* (the fungus) and sometimes other ingredients. It is salty, but its flavor and aroma depend on various factors in the ingredients and process. Some other varieties have been described as salty, sweet, earthy, fruity, and savory	East Asia	Japan	Okouchi et al. (2019)
9.	Pon ye gyi	Commonly used as a condiment or marinade, especially in dishes alongside other beans. It is traditionally made from beans	Southeast Asia	Myanmar	https://wikivisually.com/wiki/Pon_ye_gyi
10.	Tauco	This is a paste used in cuisines. Tauco is made by boiling yellow soybeans, grinding them, mixing them with flour and fermenting them in order to make a soy paste. The soy paste is soaked in salt water and sun-dried for several weeks, furthering the fermentation process, until the color of the paste has turned yellow-reddish	Southeast Asia	Malaysia	Nandiyanto et al. (2018)

(*Continued*)

TABLE 11.1 (Continued)

Sr. no.	Name of the fermented paste	Characteristics	Region	Country	References
11.	Tianmianjiang	Also known as sweet bean sauce, sweet flour sauce or sweet wheat paste, is a thick, smooth, dark brown or black paste with a mild, savory or sweet flavor	East Asia	China	Zhang et al. (2014)
12.	Tu'o'ng	It is a paste made from fermented soybeans, which is popular in vegetarian meals	Southeast Asia	Vietnam	Thanh et al. (2018)
13.	Tungrymbai	A fermented soybean food indigenous to the and Jaiñtia tribes of Meghalaya, India. Tungrymbai is usually prepared by crushing the fermented beans until it almost becomes a paste and fried in mustard oil with onion-ginger-garlic paste, black sesame seed paste, aromatics and pork	South Asia	India	Mishra et al. (2019)
14.	Doenjang	It is a traditional Korean fermented soybean paste (meju) and brine (salt solution). Meju is prepared by soaking, cooking, and crushing soybeans, followed by modeling into solid blocks of defined shape and size where consortia of naturally occurring microbes (bacteria and molds) grow with time	East Asia	Korea	Bahuguna et al. (2019)
15.	Crab paste	Crab paste is an aromatic condiment made by cooking ground crabs with oil and spices. It is a salt and liquor saturated product of fresh swimming crab, *Portunus trituberculatus*	Coastal Area	China	Chen et al. (2016)
16.	Belacan	This is a shrimp paste that is a strong-smelling; salty pink paste commonly used as a cooking ingredient	Brunei	Brunei	Kim et al. (2014)
17.	Fermented Broad Bean Crab paste	Broad bean paste is a Chinese traditional aliment which is usually manufactured via fermentation by *Aspergillus oryzae* and various groups of microorganisms with broad beans and chopped chilies as raw materials	China	China	Niu et al. (2018)
18.	Padaek	Padaek is a salt-fermented freshwater fish product popularly used in Laos as a shelf-stable all-purpose seasoning	Laos	Laos	Marui et al. (2020)
19.	Fermented Silver Carp Crab paste	Silver carp (*Hypophthalmichthys molitrix*), a freshwater white flesh fish species mostly cultivated in China and consumed in the form of fermented paste	China	China	Kasankala et al. (2011)
20.	Kutukutu	Kutukutu is the product of fermented corn pastes by natural fermentation of grains soaked in water and ground; this is an artisanal transformation process of maize	North Region	Cameroon	Roger et al. (2015)
21.	Kapi	Kapi is a fermented shrimp paste produced in Thailand	Thailand	Thailand	Phewpan et al. (2020)
22.	Fish Miso	Fish miso is used as a condiment or seasoning to add flavor to food, or in some cases to complement a dish	Japan	Japan	Giri et al. (2012)
23.	Dajiang-meju	Dajiang-meju have been used as major ingredients for the preparation of traditional spontaneously fermented soybean paste	Northeast China	China	Xie et al. (2019)

(*Continued*)

TABLE 11.1 (Continued)

Sr. no.	Name of the fermented paste	Characteristics	Region	Country	References
24.	Beitang Shrimp Crab paste	Beitang shrimp paste is fermented by different parts of shrimp, such as the head, meat, or the whole shrimp	Beitang, Tianjin	China	Yao et al. (2021)
25.	Ogi	Ogi is a cheap and readily available health-sustaining fermented food in Africa	Nigeria	Nigeria	Olaniran & Abiose, (2019)
26.	Panjin Shrimp Crab paste	Panjin shrimp paste is a famous traditional fermented aquatic product in China, especially pastes that are fermented from grasshopper sub shrimps	Panjin	China	Sang et al. (2020)
27.	Fermented Olive Crab paste	"Taralli" (Italian snack food) are produced by adding 20% of fermented olive paste from black olives	Italy	Italy	Durante et al. (2019)
28.	Pla-ra	Pla-ra is popularly used as an all-purpose seasoning in Thai cooking	Thailand	Thailand	Marui et al. (2015)

11.3 Microbial diversity as inoculum

LAB are the key microorganisms in bacterial community succession, metabolite changes, and assesses relationships between bacterial taxa and metabolites during the fermentation process of several fermented pastes such as Dajiang, Gochujang, Terasi, Huanjiang, Kinema, Miso, doubanjiang-meju, and others; hence, LAB are involved or used as the starter culture for the production of these traditional fermented pastes. Along with LAB some fungi are also used in fermentation process. Some of the commonly used LAB and fungi verities are *Lactobacillus, Pedicoccus halophilus, Enterococcus, Lactococci, Tetragenococcus muriaticus, Tetragenococcus halophilus, Arthrobacter, B. subtilis, Agrobacterium, A. oryzae, Saccharomyces cerevisiae, Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus fermentum, L. plantarum* and *Rhizopus oligosporus*. There are various fermentation process and conditions that are linked with different types of pastes. Every paste requires certain conditions to be fermented and have its own uniqueness and cultural value. The fundamental procedure of fermented paste making remains the same for most of the products, but as mentioned before every paste is having their own specific conditions which are: pH, incubation time, optimum temperature, type of carbohydrate source or salt, starter culture and other ingredients. Microorganisms with applications in fermentation as starter cultures include the following:

1. *Lactococci*, The use of lactococci is widespread and has the longest tradition in industrial starter culture technology. The principal concern of the dairy industry is the reliability and stability of these starter cultures. Many of the desirable traits of the lactococci are unstable because they are plasmid mediated. Extensive use of single strain cultures resulted in problems with bacteriophage. Genetic studies on lactococci have focused on the lactic fermentation, casein breakdown, diacetyl production from citrate and resistance to phage attack. Production of inhibitory substances (bacteriocins) by LAB is an area of increasing interest. Because a bacteriocin-producing strain could dominate the mixed cultures used for cheese making to the detriment of the fail-safe, multiple strain starter system, selection of starter strains has been against production of nisin or other antagonistic substances. With the development of phage resistant starter strains, the use of single strain starter cultures is becoming a reality and bacteriocin production may well be viewed as an asset in dairy starter culture technology in the future.
2. *Enterococcus*. The taxonomy of this group of bacteria has been vague. There are no phenotypic characteristics that separate the genus from the other genera of gram-positive, catalase-negative cocci; in fact, phenotypic identification is generally by reverse identification (Devriese et al., 1993). Enterococci produce L (+) lactic acid homofermentatively from glucose and also derive energy from degradation of amino acids. The application of enterococci for food and public health microbiologists is related to their enteral habitat. They are use as indicators for food safety and their possible involvement in foodborne illness (Stiles, 1989). The value of enterococci as indicators of fecal contamination of foods is limited by their ability to survive in the extra-enteral environment, their relatively

high heat resistance, and the fact that they can dominate the microbial population of heat-treated foods. Enterococci are also used as starter cultures in some foods and are commercially available as probiotics for prevention and treatment of intestinal disorders of humans and animals (Lewenstein et al., 1979). In particular, *Enterococcus faecium* is associated with the fermentation of a number of southern European cheeses and is often applied in their processing.

3. *P. halophilus* This microorganism is important in the fermentation of soy moromi to produce soy sauce. It requires NaCl for growth in 18% NaCl. In phylogenetic studies of the genus *Pediococcus* using 16s rRNA sequence analysis (Collins et al., 1990), it was shown that *P. halophilus* is clearly separated from the other pediococci, and a new genus *Tetragenococcus* was proposed for this organism.
4. *Lactobacillus* The classical division of the lactobacilli was based on their fermentative characteristics which includes obligately homofermentative, facultatively heterofermentative; and obligately heterofermentative. This division suited the interests of food microbiologists. Several lactobacilli of groups 1 and 2 and some of the heterofermentative group 3 lactobacilli are either used in fermented foods, but group 3 is also commonly associated with food spoilage. In *Bergey's Manual of Systematic Bacteriology,*Sneath & Jones, 1986) the genus *Lactobacillus* was described with a heterogeneous group of "regular nonsporing gram-positive rods."
5. *T. muriaticus*. This is a species of moderately halophilic lactic acid, histamine-producing bacteria. The optimum pH that supports the growth is 7−8 and the optimum temperature for growth is 25°C−35°C. *T. muriaticus* prefers NaCl concentrations of 7%−10% and tolerates up to 26% NaCl.
6. *T. halophilus* This is a gram-positive lactic acid bacterium which flourishes in extreme salt environments. Halophilic fermentation control the production of soy sauce and fish sauce. Optimum pH and temperature for growth are 7−8 and 25°C−35°C respectively. *T. halophilus* has a high salt tolerance (up to 26% NaCl).
7. *Arthrobacter*. This is an obligate aerobic bacterium that can be characterized by rod-coccus growth cycle (Gobbetti et al., 2014). Two *Arthrobacter* species namely *Arthrobacter nicotianae* and *Arthrobacter globiformis* can perform nitrate ammonification and lactate-, acetate-, and ethanol-producing fermentation processes for anaerobic growth (Eschbach et al., 2003). The optimum pH and temperature for growth are 7.2 and 25°C−28°C, respectively.
8. *B. subtilis*. This is a gram-positive, rod-shaped bacteria which can perform fermentation as an anaerobic mode of respiration. Hence, *Bacillus* are used in traditional fermentation processes as a starter culture. The optimum pH and temperature for growth are 8 and 34°C−37°C, respectively.
9. *A. oryzae*. This filamentous fungus is widely used in East Asian traditional fermented products making such as Doenjang, Fish Miso, Tianmianjiang, Tuong and Doubanjiang. Its growth is rapid and it secrets amylase which is an enzyme that catalyzes the hydrolysis of starch into sugars. The optimum pH and temperature for growth are 5−6 and 32°C−36°C, respectively.
10. *S. cerevisiae*. This is a fungus that is widely used in making various fermented products such as fermented olive paste. It has a short generation time and can easily be cultured, which makes it suitable to use as a low-cost specimen in fermentation. The optimum pH and temperature for growth are 4−6 and 32.3°C respectively.
11. *L. mesenteroides*. This is a facultatively anaerobic, gram-positive, nonmotile, nonsporogenous, and spherical species of LAB which is widely used for fermentation. *L. mesenteroides* grows best at 30°C, but can survive in temperatures ranging from 10°C−30°C. Its optimum pH is 5.5, but can still show growth in pH of 4.5−7.0 .
12. *R. oligosporus*. Tempeh is a traditional Indonesian food made from soybeans that have been fermented, or broken down by microorganisms. Culture of R. oligosporus is the preferred starter culture for tempeh production for several reasons. It grows effectively in the warm temperatures at 30°C−40°C. It exhibits strong lipolytic and proteolytic activity, creating desirable properties in tempeh (Table 11.2).

11.4 Production strategies and biochemistry of fermented paste

Production of pastes is usually conducted through fermentation processes using various forms of raw materials depending on the type of paste required. Globally, pastes are produced locally depending on the country or region. In China, doubanjiang is produced as a traditional fermented red pepper paste from red pepper paste (*Capsicum annum* L.), broad bean (*Vicia faba* L.), wheat flour and salt.The traditional manufacturing process of doubanjiang consists of fermentation of broad beans with salt (12−14% w/v) to make doubanjiang-meju, fermentation of red peppers (approximately 1−2 cm) with salt (14−16% w/v) to yield red pepper moromi, and aged fermentation for more than 6 months in the natural environment of a mix of doubanjiang-meju with red pepper moromi at a ratio of 4:6 to improve flavor and taste characteristicsBroad beans paste has been produced through fermentation using broad beans as raw materials with *A. oryzae* as fermenting microorganism. The initial step of broad bean paste production is the steeping and boiling of

TABLE 11.2 Morphological and biochemical diversity of dairy important microbes used in preparation of fermented paste.

Sr. no	Scientific name	Name of fermented paste	Characteristics	Optimum pH	Optimum temperature (°C)	Nutritional requirement	References
1.	*T. halophilus*	Padaek, Pla-ra, Terasi, Kapi	Gram-positive lactic acid bacterium which flourishes in extreme salt environments. Used in halophilic fermentation processes such as the production of soy sauce and fish sauce.	7–8	25–35	*T. halophilus* has high salt tolerance (up to 26% NaCl)	Marui et al. (2015, 2020), Phewpan et al. (2020)
2.	*Tetragenococcus muriaticus*	Padaek, Pla-ra, Terasi, Kapi	*Tetragenococcus muriaticus* is a species of moderately halophilic lactic acid, histamine-producing bacteria	7–8	25–35	*Tetragenococcus muriaticus* prefers NaCl concentrations of 7–10% and tolerates up to 26% NaCl	Marui et al. (2015, 2020), Phewpan et al. (2020).
3.	*Staphylococcus gallinarum, Moraxella catarrhalis, Micrococcus luteus* and *Photobacterium angustum*	Crab paste, Panjin shrimp paste		6–7 7	30–3733–353730–32		Chen et al. (2016), Huang et al. (2011), Sang et al. (2020)
4.	*Arthrobacter*	Crab paste		7.2	25–28		Chen et al. (2016)
5.	*Sphingobacterium*	Crab paste		7	40–45		Chen et al. (2016)
6.	*Bacillus*	Crab paste, Dajiang, Belacan, Ogi		8	34–37		Chen et al. (2016), Kosisochukwu et al. (2018), Xie et al. (2019)
7.	*Psychrobacte*	Crab paste, Panjin shrimp paste		7–9	20–30		Chen et al. (2016), Sang et al. (2020)
8.	*Agrobacterium*	Crab paste		7	28		Chen et al. (2016)
9.	*Salinivibrio*	Crab paste		8–8.5	37		Chen et al. (2016)
10.	*Micrococcus*	Crab paste		5.5	25–37		Chen et al. (2016)
11.	*Kocuria rosea*	Crab paste		7	25–37		Chen et al. (2016)
12.	*Corynebacterium*	Crab paste		7–8.5	25–37		Chen et al. (2016)

(*Continued*)

TABLE 11.2 (Continued)

Sr. no	Scientific name	Name of fermented paste	Characteristics	Optimum pH	Optimum temperature (°C)	Nutritional requirement	References
13.	*Rhodococcus*	Crab paste		7–8	30		Chen et al. (2016)
14.	*Aspergillus oryzae*	Tuong, Doubanjiang, Tauco, Fermented Silver Carp Paste, Doenjang, Fish Miso, Tianmianjiang	*Aspergillus oryzae* is a fungus widely used in traditional Japanese fermentation industries.	5–6	32–36		Giri et al. (2012), Jang et al. (2014), Kasankala et al. (2011), Nandiyanto et al. (2018), Thanh et al. (2018), Zhang et al. (2014, 2020)
15.	*Bacillus amyloliquefaciens*	Tuong		7	30–50		Thanh et al. (2018)
16.	*Enterobacter mori*	Tuong		7	35		Thanh et al. (2018)
17.	*Jeotgalibaca*	Panjin shrimp paste		8	30		Sang et al. (2020)
18.	*Jeotgalicoccus*	Panjin shrimp paste		7–8	30–35		Sang et al. (2020)
19.	*Lysinibacillus*	Panjin shrimp paste		7–8	37		Sang et al. (2020)
20.	*Sporosarcina*	Panjin shrimp paste		6.5–8	20–30		Sang et al. (2020)
21.	*Rhizopus*	Dajiang-meju, Doenjang		7–8	25		Jang et al. (2014), Xie et al. (2019)
22.	*Penicillium*	Dajiang-meju, Dajiang		5–9	24		Xie et al. (2019)
23.	*Geotrichum*	Dajiang-meju		5–5.5	25		Xie et al. (2019)
24.	*Saccharomyces cerevisiae*	Fermented Olive Paste		4–6	32.3		Durante et al. (2019)
25.	*Leuconostoc mesenteroides*	Fermented Olive Paste		5.5	30		Durante et al. (2019)
26.	*Lactobacillus brevis*	Kutukutu		4–5	30		Roger et al. (2015)
27.	*Lactobacillus fermentum*	Kutukutu		5.5	25		Roger et al. (2015)

28.	*Lactobacillus plantarum*	Kutukutu		6	30		Roger et al. (2015)
29.	*Lactococcus*	Kutukutu		6.3–6.9	30		Roger et al. (2015)
30.	*Streptococcus*	Kutukutu		6.5	37		Roger et al. (2015)
31.	*Leuconostoc*	Kutukutu		6–6.5	26		Roger et al. (2015)
32.	*Tetracoccus*	Dajiang		7–7.5	25–35		Xie et al. (2019)
33.	*Aspergillus*	Dajiang		5.48	37		Xie et al. (2019)
34.	*Rhizopus oligosporus*	Tauco		5.5–5.8	25		Nandiyanto et al. (2018)
35.	*Lactobacillus futsaii*	Pla-ra		5–6	37		Marui et al. (2015)
36.	*Lactobacillus acidipiscis*	Pla-ra		Below 5	37		Marui et al. (2015)
37.	*Lactobacillus rennini*	Pla-ra		3.7–4.5 and 8	37		Marui et al. (2015)
38.	*Pediococcus argentinicus*	Pla-ra		6	20–30		Marui et al. (2015)
39.	*Weissella paramesenteroides*	Pla-ra		8	25–37		Marui et al. (2015)
40.	*Halanaerobium fermentas*	Pla-ra		7.5	35		Marui et al. (2015)
41.	*Clostridium*	Pla-ra		5–9	43–47		Marui et al. (2015)
42.	*Sphingomonas*	Pla-ra		7	28		Marui et al. (2015)
43.	*Bacillus subtilis*	Doenjang, Cheonggukjang, Kinema		8	34		Lee et al. (2013), Tamang et al. (2015)
44.	*B. licheniformis*	Doenjang, fermented Broad Bean paste		9–10	37		Jang et al. (2014), Niu et al. (2018)
45.	*Mucor*	Doenjang		4.5–5	25		Jang et al. (2014)
46.	*Lactobacillus*	Ogi, Tungrymbai		5.8–6	30–40		Kosisochukwu et al. (2018), Mishra et al. (2019)
47.	*Tetragenococcus*	Beitang shrimp paste		7–8	25–35		Yao et al. (2021)
48.	*Lentibacillus*	Kapi		7	30		Phewpan et al. (2020)

broad beans in water to produce fermentable sugars and other nutrients. The *A. oryzae* 3.042 strain, which is safe to use in the food industry, is then inoculated into the boiled broad beans to start the fermentation and saline water (16−18%, v/v, sodium chloride) is then added. In traditional broad bean paste, the fermentation is usually conducted in an open environment at room temperature for 30−35 days. This means, besides *A. oryzae* strain, many other groups of microbes, especially *Bacillus* species and some wild fungi, were also involved in the fermentation process . When the fermentation is finished, the fermented broad bean paste is thermally treated at 66°C for 2 days and chopped chilies are added as flavor enhancer. This mild heat treatment, serves as a preservation method in the production of broad bean paste since, actual sterilization temperatures (up to 121°C) would produce unpleasant flavors in the finished broad bean paste. Crab paste is yet another fermentable paste consumed regularly by people in the coastal area of China. This paste is a salt and liquor saturated production of fresh swimming crabs (*Portunus trituberculatus*). It is produced directly by mixing crabs meat with ingredient and liquor under air (Chen et al., 2016).

In Korean, traditional soybean paste called *doejang* is their major fermented soybean paste consumed as a condiment for preparing stew or dipping sauce for vegetables, fish and meat. Traditionally, doenjang is prepared using fermented meju and brine. Typically, meju is prepared by soaking, cooking, and crushing soybeans, followed by modeling into solid blocks of defined shape and size where consortia of naturally occurring microbes especially bacteria and molds grow with the time. Subsequently, salt solution is added to meju and fermented for few more months. Later, the solid portion is separated from the liquid and fermented for several months resulting in doenjang. The nutrient value, taste and texture of doenjang are highly dependent on fermentation conditions, basic ingredients and involvement of microorganisms. Therefore, the addition of new ingredients has high possibilities to improve the doenjang quality both in taste and functional property. Further, selection of superior quality of basic ingredients like seeds also has an impact on doenjang quality which can be achieved by utilizing many modern techniques. Extensive efforts have been made to improve the doenjang quality by altering fermentation condition, substrate, and microbial inoculum. Kochujan is another Korean traditional soybean red pepper paste. It is produced by the fermentation of red peppers, powdered meju, salt, malt digested rice syrup and rice flour for a period of 6 months. It is a fermented soybean-based red pepper paste that has long been identified as one of the most representative seasonings in Korean cuisine. The bioavailability of bioactive ingredients, such as peptides, alcohols, organic acids, capsaicin, and flavonoids, are increased during this fermentation. Either single or combined substances in Kochujang have shown biological properties for antiobesity, antioxidants, and antimutagenesis (Lee et al., 2017). Chili shrimp paste (CSP) is an exotic traditional Southeast Asian condiment prepared using mainly fresh chilies and fermented shrimp paste (belacan) which is attributed to a strong pungent fishy odor. It is prepared usually by mixing ingredients of 55% red chili (*C. annum*), 14% bird's eye chili (*Capsicum frutescence*), 17% fermented shrimp paste (belacan), 7% sugar, 2% salt (2% salt plus the salt content of belacan together made 4.4% salt in the final product), 4% calamansi juice and 1% citric acid (Cheok et al., 2017). Ogi paste is one of the most popular fermented heath foods especially in West African countries. It is produced from soghum (*Soghum bicolar*), millet (*Pennisetumm typhodenum*) and maize (*Zae mays*) (Olaniran & Abiose, 2019). These pastes were subjected to seven treatments at ambient and refrigerated temperatures for 4 weeks during which sensory analysis was carried out, and mineral content, total antioxidant activities, and proximate composition were evaluated. Ogi (maize) enhanced with 2% garlic + 2% ginger and ogi (sorghum) enhanced with 4% garlic + 2% ginger were most preferred.

11.4.1 Fermented shrimp paste

Thai conventional fermented paste which is made by shrimp is generally called Kapi. Kapi is customarily arranged from mysid shrimp blended with salt at a ratio of 3−5:1 and afterward dried in sunlight to diminish the moisture, lastly it is crushed into a uniform paste. The time of fermentation is to age for 2−6 months to create attractive and extraordinary tastes and unique odors.

11.4.2 Soybean paste

Soybean paste otherwise called doenjang is fundamentally prepared with meju and set up by drenching, steaming, and pulverizing the soybeans. At that point, paste moisture is removed, and hung up with rice straw for 1−3 months for the development of characteristic microorganisms. That aged meju is brined and aged for more than 2−3 months.

After the fermentation process, it is isolated into two sections: the supernatant fluid and hastened strong buildup. The fluids are sifted to acquire soy sauce, and the solids are kept into earthenware and further matured for more than 2

month to make customary soybean glue. Conventionally, doenjang is aged in a common habitat, different microorganisms, for example, *Bacillus, Rizopus, Mucor*, and *Aspergillus* species aid in the fermentation process.

11.4.3 Fermented red pepper paste

Fermented Korean red pepper paste, known as gochujang, is made by fermenting a mixed paste made by red pepper powder (*C. annum* L.), glutionous rice, meju, salt and water. For the preparation, barley malt is soaked in distilled water overnight at a temperature of 20°C. Then, the mixture is filtered and glutinous rice powder is added to it while stirring. The mixture is heated in 60°C for completed the saccharification.

Salt is added to the prepared solution and boiled for 30 minutes. After boiling it is cooled to 40°C, and then meju powder and red pepper powder are added to it and mixed properly with rice paste. Finally, the mixture is kept for fermentation at 25°C for 90 days in an aerobic incubator.

11.4.4 Fermented fish paste

11.4.4.1 Fermentation method 1

Koshihikari rice (*Oryza sativa*) which is made in Japan, absorbs two volumes of new water for 12 hours at normal room temperature and steamed at 90°C for 1 hour. In the wake of cooling to room temperature, the rice is immunized with the koji shape, hatched at 35°C for 48 hours, and subsequent malt-rice was utilized as koji, the starter for aging. New fishes were guillotined, gutted, washed, cleaned, and deboned utilizing a model NF2 deboning machine outfitted with a drum containing holes (4 mm with distance across).

The fish were ground independently with a model M-22 processor (Nantsune Tekko, Osaka, Japan) and afterward put into an aluminum-covered polyvinylchloride pocket, which was vacuum-fixed and steam-warmed at 90°C for 1 hour. Parts were then channel squeezed at 2 MPa to accomplish moisture somewhere in the range of 50%−55% by utilizing a model KS-1 channel press (Komagata Kikai Seisakusho, Tokyo, Japan). The subsequent dried-out meat was washed multiple times with five volumes of freshwater before squeezing. Koji, fish, and salt were blended in a processor at a ratio of 5:5:1 to wet weight. Roughly 3 kg of fish paste was stuffed into a 5-L plastic holder and matured in the range of 25°C−30°C for 90 days. The substance of every compartment was blended together once per month. The readied material, named fish miso, signifies "matured fish paste" in Japanese. Following 90 days of maturation, fish miso items are put away at two unique temperatures—10°C and 25°C. The items are inspected at 0, 15, 30, 60, 90, 135, 180, 270, and 365 days for examination.

11.4.4.2 Fermentation method 2

Fermented fish paste is prepared using dried anchovy fish set up with 2% of sodium chloride(NaCl) (w/v), and allowed to ferment at normal room temperature for different days of fermentation period. Fish used as crude material and sunlight-based salt acquired at a nearby market in Semarang. ninety-eight percent of fish and 2% of sun-oriented salt were completely blended then pounded. The crushed salt and fish mixture was dried using the sun until proper texture, nonclingy, and afterward granulated once more. Semi-dried fish paste was aged for 2 days at encompassing temperatures. Semi-dried paste was framed into tube-like structures 3 cm length across, 10 cm long and dried by the sun for 2 days, and afterwards, wrapped firmly in banana leaves. Then kept it until the fermentation process completed. Tests were vigorous for tangible with synthetic examinations (amino acids and unsaturated fat) on different days maturation.

11.4.5 Fermented black garlic paste

Black garlic paste preparation, is prepared from nonharmful and organic white skin garlic with proper cleaning and drying. The mixed bacteria liquid solution is prepared with the mixed bacterium leavening agent and pure water, mixed with weight 3%−4.5% with fermentation nutrient solution. The mixture is covered and kept at 35°C−41°C for 8 hours. Temperature is adjusted to 20−24°C for 36−50 hours for slow fermenting. The mixture is then heated to 100°C for 3−5 minutes nd then left for 1 week at room temperature. After this step the fermentation process is complete and the mixture is allowed to dry naturally for 1−2 days in a shady and cool ventilated place will mature the product for consumption.

11.4.6 Fermented milk tomato paste

Red color and round-shaped raw tomatoes are selected. Two kilogram of tomatoes are used for the paste preparation. The raw materials are washed and dried properly, then steamed at 110°C for 10 minutes. The tomato rind is removed and crushed with a blender.

Yogurt, or probiotic fermented milk, is prepared by mixing 1 liter of fresh milk with 25 mL plain yogurt. Milk is pasteurized by heating 90°C for 15 minutes. Mixture is feremented for 24 hours at normal room temperature at 30°C.

11.5 Methods of investigation of fermented compounds/sensory characteristics or drivers of liking

These pastes are appreciated as uniquely flavored seasoning for many centuries in various countries. All fermented paste were evaluated on the basis of aroma quality. A series of complicated processes, the ingredient source, formulation, fermentation method, length, and condition can all affect the sensory quality of these fermented pastes. Factors that positively or negatively influence the liking for a food product of interest is one of the key components that leads to the development of a successful product. Food acceptance is often heavily influenced by sensory perception, because acceptance is considered an output of brain processing based on the sensory property input from food consumption.

11.6 Conclusion

In many countries, fermented paste is used as a side dish or a dressing. Each day, people make many pastes with new recipes. The most popular countries that are involved in the preparation of fermented pastes are Korea, Thailand, China, and Japan. The reason for this could be the usage of spices in the foods by Asian people more than western countries. These countries have used spices in their paste not only to enhance the flavor but also to increase the shelf life. During fermentation, selection of different processed sugar isslected many health benefits. The fermentation process need to be showing high metabolic activity of a microorganism toincrease the shelf life of the food naturally. Many microorganisms yet need to be used for there involvementin the fermentation process. Different fermented pastes have different fermentation conditions with cultural values. They possess different tastes and can be classified as vegetarian and nonvegetarian products. Some examples for fermented pastes are shrimp, soybean, red pepper, and fermented fish. Fermented paste benefits people not only as a food product but also creates an environment harmful for pathogens.

Spicy fermented pastes are very popular as flavoring condiments worldwide. Fermented pastes are the result of fermentation reaction triggered by dairy microbes. Spicy fermented paste condiments are especially popular in many countries. Consumption of the fermented paste is gradually expanding around the world, due to their various health benefits. A lot of aspect related to microbiology, biochemistry, macro ingredients utilized, processing methods, and sensory characteristics used in production of these fermented pastes. Beyond nutrition, fermented paste reportedly provides appetizing options towards the potential therapeutics and prevention of various diseases including cancers, osteoporosis, and cardiovascular diseases.

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Chapter 12

Chemistry and material studies in fermented dairy products

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12.1 Introduction

Milk is observed as a foundation of complete nutrition, which is generally required for both infants as well as adult consumers. Although milk can be consumed without processing, the variety of dairy foods manufactured from milk after processing are of utmost significance and can be consumed throughout life. When uncooked milk is held in reserve at ambient temperatures, on the way it undergoes bacteriological decay, which after a few days turns milk bitter in taste. This commonly results because the naturally present LAB, or lactic acid bacteria, is an extraneous contaminant in the milk (Mehta, 2015). The LAB subsequently grows in it and produces lactic acid that supports in coagulating the liquid. Likewise, various other flora of bacteria or microorganism may grow, that can be intentionally introduced to raw liquid, in order to obtain a desirable flavor, taste, texture and most importantly, to increase the shelf-life of dairy foods. Numerous types of fermented dairy goods such as kefir, yogurt, dahi, buttermilk, acidophilus liquid, sour milk, varieties of cheese etc., are prepared around the globe, mostly by lactic acid fermentation (e.g., yogurt and dahi), but sometimes a mixture of LAB and yeast (e.g., kefir) can also be engaged in fermentation i.e., alcohol fermentation, to obtain special characteristic features in fermented dairy foodstuffs. Other microorganism (molds) and starter cultures are developed and are involved in production of large variety of fermented dairy foods. Traditionally, cheeses were prepared by farmers on a small scale from raw milk of cows, goats, or ewes, with the naturally present LAB in milk. A day prior to the making of cheese or additional fermented dairy foods, the cultures of such microbes were developed by incubating the milk or keeping under controlled conditions. The use of this processing technique of cheese production is still common in many European countries. Now, the selected meze cultures are involved in the production of fermented dairy goods, which imparts the uniformity, specific texture, and characteristic flavor and taste, to the certain fermented dairy foods and variety of cheeses (Wouters et al., 2002). These fermented dairy foods are well consumed because of their characteristics acidic taste, good digestibility, health promoting benefits, and their proximity to functioning foods, which are in demand today. Fermentation is the primogenital technique of conserving milk solids with over 3500 traditional fermented dairy foods existing all over the world with huge benefits to humans. The nature (flavor, texture, aroma, etc.) of fermented dairy foods depends principally on the type of milk utilized, variety of breed, manufacturing process and condition, fermentation temperature and climate, and subsequently on other treatments involved for its production.

12.2 Fermented dairy foods

All milk or milk-derived components, when undergoing fermentation yield fermented dairy foods. The variety of high nutritive fermented dairy foodstuffs are prepared by means of an extensive variety of bacteria or microbes merged in starter culture. Lactic acid, particularly thermophilic LAB and other microorganisms are eminent for fermentation process (Wouters et al., 2002). These can be either naturally present in milk or can be externally added as a specific

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culture. In the commercial manufacturing process, several starter cultures like yeast (e.g., kefir grains), molds, or an amalgamation of LAB and other microbes are utilized for the making of fermented dairy foods which converts lactose into lactic acid and added substances existing in milk. The configuration of lactic acid produced i.e., L-form and D-form, largely depending on the type of culture or microorganisms involved. The starter cultures plays a dynamic part in fermentation. This reasons as its major functions are preserving the derived product with a longer shelf-life and imparting increased safety, providing high benefits to human health, improving the sensory and rheological assets of dairy goods, and allowing the manufacture of bacteriocins as great food preservatives (Bhullar et al., 2002; De Vuyst & Vandamme, 1994; Tamime, 2006). Based on the type of bacteria or microorganisms involved, the process of fermentation carried out is either homo-fermentation through the glycolysis pathway yielding solely lactic acid; or hetero-fermentation through the pentose phosphate pathway yielding lactic acid along with carbon dioxide and acetic acid; or through both pathways (Mehta, 2015). Furthermore, the type of bacteria or microorganisms involved greatly influence the taste, flavor, and texture of fermented dairy foods. Thermophilic lactic acid formation plays a significant role in bringing down the pH, which offers a great nutritive and preservative advantage to fermented dairy foods than original milk (Wouters et al., 2002). Additionally, L-lactic acid offers great digestive property and is easily metabolized by humans, whereas higher consumption of D-lactic acid may effect in blood improvement and hyperacidity in urine. The IDF or International Dairy Federation defines fermented dairy foods as, "the foods organized from milk and/or milk consequent elements, that have been at least pasteurized by the action of precise bacteria that outcomes in a lessening of pH and clotting of casein. The starter culture used should be feasible, lively, and plentiful having at least 10^7 CFU/gm" (IDF, 1988, 1992).

12.3 Role of chemistry in fermented dairy foods

The part of fermented dairy foods in human nourishment is well familiar and the qualities of these goods were recognized to human even throughout the prehistoric days of evolution. These goods have extended a vital element of nutritious food. The therapeutic and alimentary belongings of several incited nourishments have been practiced by numerous age groups. The technical public offered motivation to these principles in 1910, once Eli Metchnikoff recommended that humans must drink milk fermented with lactobacilli to lengthen his lifetime. He hypothesized the needed microorganisms in Bulgarian milk that might aid in overpowering the unwanted microbes in the intestine of humans. The opinion showed the technique for discovering the possibilities of lactic cultures and cultured foods in the mitigation of human and animal complaints. Lately, reputation has been specified to yield fermented milk with enhanced fitness qualities as the chief healing abilities of these goods. A fermented milk product has been well-defined by the IDF as a milk product made from skimmed milk or not, with precise cultures. The microbes is reserved thriving until sold to the costumers and may not comprise any infective origins. The managed foods are steadied by considerably abolishing or deactivating all existing enzymes. This deactivation, habitually done by blanching, is particularly pointed at enzymes that tempt off-flavor growth through storing treated foods. Another such enzyme is peroxidase. When blanching is approved to the opinion where peroxidase action is significantly demolished, maximum amount of the other enzymes existing in produces are also devastated or disabled. In supplementary confrontations, in disabling the enzymes that are harmful to diet steadiness, the enzymes intricate in flavor establishment are also pretentious. The new flavor of the diet is changed or partly ruined by dispensation since many flavored complexes are instable or heat-labile. Flavor forerunners seem to live dispensation, thereby on behalf of a possible or hidden foundation of new flavor. The predecessor and enzyme education may also offer data for selective obliteration or control of enzymes and/or predecessors answerable for unwanted flavors formed through storing of food goods (Hewitt et al., 1956, 1957).

12.4 Material studied in fermented dairy foods

Fermentation is the primogenital biotechnological technique to preserve veggies; it is accountable for many favorable food characteristics, for example, flavor, shelf-life, and quality. Numerous types of fermented vegetable goods occur in the biosphere (e.g., soy sauce, miso, kimchi and pickled vegetables), but their profitable supply delays fermented meat and dairy goods due of a lack of consistent production procedures, joined with their elements being founded on preventive and changeable climate and topographical settings (Cetin, 2011). There exists a vast variety of milk and fermented dairy foods depending on the fermentation process, use, composition, stage of preparation, and starter culture and bacteria involved during its preparation. Some common materials studied in fermented dairy foods are discussed next.

12.4.1 Yogurt

Yogurt is the most popular fermented milk food, and it is universally widespread (Benkerroum & Tamime, 2004). The term "yogurt" originated after the Turkish word yoğurt (Moore, 2004), which is formed from the words, *yoğ urmak* meaning "to knead" and *yoğun* meaning "dense" or "thick" (Peters, 2004). Yogurt is prepared by incorporating certain specific bacteria strains or starter culture into the milk, which subsequently ferments the milk under controlled temperatures of about 42°C−43°C and providing specific ecological situations in fermentation reservoir (in industries) or in homemade vessels. The bacteria in the milk convert lactose into lactic acid, produced as a left-over artefact, that raises the overall tartness of the product formed. Due to the increased acidity by lactic acid, the process of denaturation proceeds, in which the milk proteins starts to coagulate and turns to a solid mass, known as curd (Robinson & Tamime, 1986). Fermented milk/dairy product may be called pasteurized foods having no living bacteria in it. Similarly, once yogurt is sterilized, its chief goal is still to slaughter injurious microbes, subsequently it slays great quantities of important microorganisms also, such as *Lactobacillus rhamnosus, Bifidus*, and *Acidophilus*. Yogurt is a semisolid fermented milk product. Its fame has full-grown and is today consumed over most of the globe. Though the constancy, flavor, and fragrance may differ from one area to another, the rudimentary elements and manufacturing are fundamentally reliable (Robinson, 2007).

12.4.2 Kefir

An old-style, fermented drink/brew, kefir is formed after fermentation of milk by kefir grains. It has an acidic taste and creamy texture. The term kefir originated after the Turkish word *keyif*, stating "good feeling" and *kef* meaning "pleasant taste" in Turkish, which refers to the feelings and taste that is experienced after drinking it (Leite et al., 2015; Prado et al., 2015). Kefir particles are white to yellowish in color that appears to be cauliflower in shape, with a smarmy touch, but fixed surface. These grains have a multifaceted bacteriological arrangement of *Lactobacilli, Lactic streptococci*, LAB species, yeasts, and acetic acid bacteria, trapped in dense, inert medium made up of polysaccharides and proteins (Kalamaki & Angelidis, 2017; Macuamule et al., 2016). The general bacteria from kefir particles from diverse areas may have variances among them. The microbes of the particles are typically numerous homo- and heterofermentative LAB species of *Lactococcus, Lactobacillus, Streptococcus*, and *Leuconostoc*; acetic acid bacteria species of Acetobacter. In Taiwanese kefir grains, *Lactobacillus* was the utmost common type spotted, and Lb. *kefiri* was the maximum often noticed species (Chen et al., 2008). Kefir is fermented milk (drink) formed by acetic acid bacteria, yeasts, and LAB, stuck in a multifaceted matrix of polysaccharides and proteins (Simova et al., 2002). The difference in kefir drink from added fermented milks, is in its starter culture, which in this case, exists in the form of "grains." The grains have a definite assembly and act as physically dynamic creatures. Outside its intrinsic high nutritious value as a basis of protein and calcium, kefir has a long custom of being known as virtuous for fitness in states where it is a essential in the food. Many studies have showed that kefir greatly benefits in the treatment of un-normal stools to normal over a shorter period of time (Bukhgalter, 1974; Murofushi et al., 1986; Orlova et al., 1980). Kefir, when administered orally, improves the exact mucosal immune rejoinder against cholera holotoxin in young grown-ups (Thoreux & Schmucker, 2001). The source of kefir's health promoting effects is indistinct, that whether these health endorsing properties are due to one specific bacteria or yeast (Yuksekdag & Aslim, 2004), or due to peptides released through milk fermentation or whether it is a synergic blend of these influences.

12.4.3 Dahi (curd)

Along the countryside, dahi (fermented milk curd or Indian yogurt) is thought to be the most prevalent and eldest fermented dairy product, and is actuality used as a unvarying piece of the food. Its use for the treatment of diarrhea was stated in the Ayurveda prose from about CE 600. Fat free curd/dahi (buttermilk) has been used to treat long-lasting gastrointestinal illnesses like chronic diarrhea, colitis, etc., by Indian physicians. In addition to so many benefits of dahi for digestion, it, when taken along with a usual diet, may aid in reducing the cycle and number of episodes as well as duration of diarrhea (Ullmann & Korzenik, 1998). Dahi is prepared by lactic acid fermentation of buffalo or cow milk, in a similar fashion to yogurt, and is considered as a functional food due to its nutritional and therapeutically values (Abbas & Jafri, 1992). Dahi has a typical chocolate shade, smooth texture with firm consistency, a cooked and caramelized flavor. The strong heating results the milk to coffee brown. It is made in clay vessels which are effortlessly accessible, inexpensive, and utilized as one time vessel that gives the item a "muddy" essence and profuse texture equated with dahi made in elastic bottles, thus making it extremely widespread amid customers. Regrettably, the earthenware pots

are progressively being substituted with plastic flasks in city ranges. It is produced from cow and buffalo milk or from a combination of them by a old technique by means of native non-descriptive starter culture (previously made dahi) comprising LAB and other fermentative animals. It is assisted as a sweet after the distinctive Bangladeshi pulao dish that is extremely nourishing and rich. It is the found that dahi supports in absorption and cures abdominal disorders such as constipation, diarrhea, and dysentery. This recommends that it might have some antipathogenic possessions that offer safety from these disorders. LAB have the skill to quickly yield a number of antimicrobic materials, such as free fatty acids, organic acids, ammonia, diacetyl, H_2O_2 and bacteriocin, that have the ability to prevent development of a diversity of food-borne decay and infective creatures (Jack et al., 1995; Vandenbergh, 1993).

12.4.4 Acidophilus milk

Ingesting of acidophilus milk foodstuffs as a nutritional assistant is considered to communicate numerous fitness assistances. Though, *L. acidophilus* raises gradually in milk. Henceforth, the foods set by using *L. acidophilus* as the only fermenting bacterium own an unfriendly flavor. This has demoted these foods to a grouping of a medication rather than a constituent of a regular food. Later, hard work have made to grow foods which own the characteristics of *L. acidophilus* and so far are able from objectionable flavors. Meanwhile, milk has been a element of our food for times, it is but ordinary that it can serve as a brilliant host to enable ingestion of an animal. In adding to being broadly disbursed in foods, such as yogurt and ice cream, dairy products have also been used as carriers of this creature (Mital & Garg, 1992). Among the possible benefits to health or nutrition from consuming milk containing lactobacilli or *Bifidobacteria* include (1) control of intestinal infections, (2) anticarcinogenic activity, (3) improved lactose utilization, and (4) aid in controlling serum cholesterol levels. These represent the four main areas that have been addressed with regard to possible benefits derived from these dietary cultures. Many factors govern whether or not a dietary culture can benefit health and nutrition. Consideration of the cultures involved shows tremendous variations among strains. This combined with variations among persons makes very complicated systems from which to evaluate potential benefits derived from the cultures (Gilliland, 1989). Milk fermented with strains of *L. acidophilus* has been recognized for countless centuries as acidophilus milk. Furthermore, dissimilar *L. acidophilus* strains are utilized in the handing out of dairy foods like acidophilus yogurt and sugary acidophilus milk. The nourishing and calming assistances derivative over consumption of dairy nutriments, comprising feasible *L. acidophilus* as a food or feed complement, have been the focus of studies for the past two decades (Salminen et al., 1996). Milk foods founded on *L. acidophilus* have met with several difficulties. Chief complications for marketplace extension throughout the globe is the sluggish development of *L. acidophilus* in milk particularly without development agents; maintenance of microbial feasibility throughout storing; comparatively high acidity; and the unappealing flavor and steadiness of the produce (Brashears & Gilliand, 1995).

12.4.5 Various types of cheese

Conventionally, cheese is gained by utilizing liquid milk into a gel. This is done by addition rennet to milk. The interstitial fluid of the gel (whey) is disqualified gradually by syneresis. Throughout syneresis, the key elements of the gel (fat and protein), steadily turn out to be more concerted and obtain the distinctive figure, consistency, and structure of the specific cheese to be made. In preparation, cheese makers do not control completely all the aspects (bacteriological, thermal, and physico-chemical) which control the technological stage of whey drainage, and the cheese, particularly the soft ripened type, is, as a rule, varied in structure, superiority, and mass. The economic consequence of this threefold heterogeneity is significant because of the safety margins (e.g., minimum weight per cheese must be above a certain legal limit) which the cheese maker must observe regarding the raw material he uses. It has been found that the lone way to diminish this heterogeneousness was to keep the milk ingredients which usually form the cheese in a homogeneous liquid form. Then it could be heated easily and rapidly or cooled and distributed in doses, each dose corresponding to a cheese such as Camembert. The process then could be made continuous. This thinking suggests that the buttermilk drainage must be done prior to clotting of the liquid (Maubois, 1973; Maubois & Mocquot, 1971).

Cheese is a tremendously multipurpose food product that has a varied variety of qualities, essences, and customers. The quality and form of cheese diversities can variety from lax to fixed, brittle to long, mechanically open to closed, smooth/creamy to curdy, or from cheese with ruptures to rounded eyes. The physiological characteristics of cheese are greatly resolved by the casein content, the type, quantity, and concentration of casein intercommunications, cheese constitution, and ripening withdrawals (Horne, 1998; Lucey et al., 2003). Cheese has been used as an element in different foods since the first reported usage of the cheese itself, principally to add essence to bland foods. Now, cheese is being used frequently as a component in a broad diversity of processed foods consisting of delicacies, soups, flavorings,

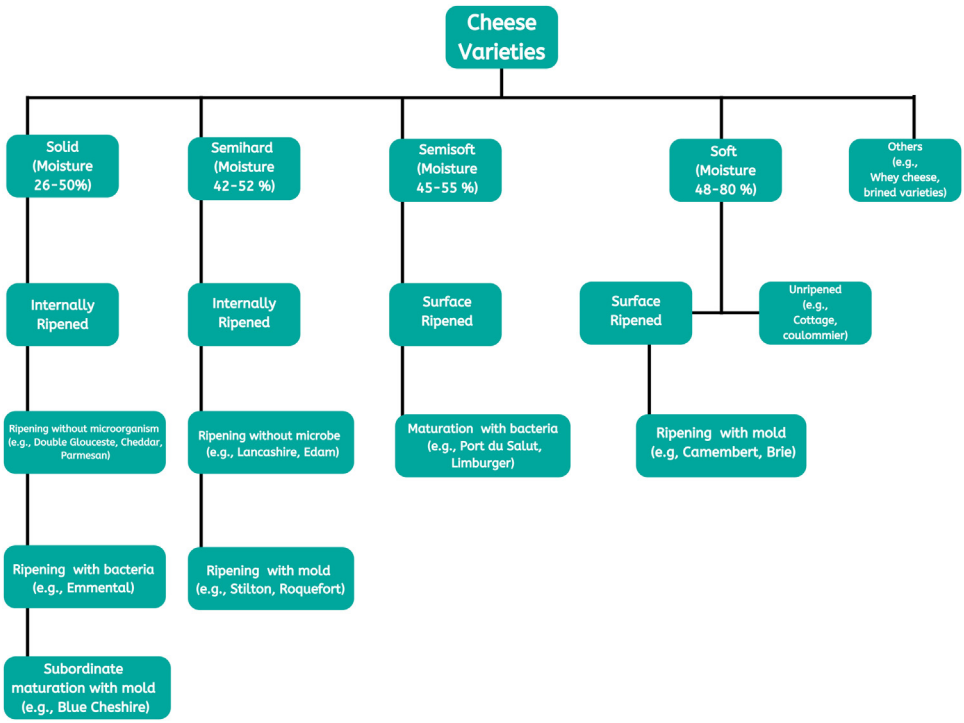

FIGURE 12.1 Classification of cheese variety according to moisture content and means of ripening (Varnam & Sutherland, 2001).

crackers, casseroles, fillings in pies and pastry as well as the general use of cheese as a layer on pizzas and as cheese pieces on burgers and cold (sub) sandwiches. There have been many wonderful reports on the functionality expected of cheese when used as a food component (Guinee & Kilcawley, 2004; Guinee, 2002). The main international variations are cheddar, Camembert, Grana types, mozzarella, Gouda, Emmental, and Quark. The classification of cheese is extremely complicated. The IDF, or International Dairy Federation, summarized different cheese categories below various sources like the nation of origin; uncooked milk (buffalo, goat, sheep, cow, etc.); kind of cheese (semi-hard, hard, soft, acid coagulated, fresh, or whey cheese); inner qualities (close or open texture; medium, small, or large eyes/holes; slit openings in curd; blue or white mold-ripened; the color of curds); external attributes (rind hard, smooth, soft, or rough, spices or herbal additions, type of coating, smear or mold-ripened,); the mass of cheese (appearances and dimensions); fat-in-dry matter/fat-on-dry basis (least or greatest percentage); water (maximum percentage); and water-in-fat free substances/moisture-in-fat free substances. Most of the cheese varieties may be described through the moisture present and according to how any maturity is achieved (Varnam & Sutherland, 2001) which is shown in Fig. 12.1.

12.5 Chemical composition of fermented dairy foods

The composition of fermented dairy foods varies considerably depending upon the raw milk of dairy cattle (e.g., cow, goat, buffalo, sheep, camel, etc.). Moreover, it is also largely affected by various factors like variation in breeds, lactation and feeding state/stage, manufacturing process, seasonal variations, number of calving's of cattle (parity), age and health of cattle, frequency of milking per day and several other environmental factors during its production (Bansal et al., 2003; Jenkins & McGuire, 2006; Laben, 1963; Walker et al., 2004). Additionally, the compositional and organoleptic qualities of fermented dairy foods varies depending on: (1) initial quality of milk, (2) manufacturing condition, (3) types and levels of starter cultures, and (4) age of foods. Since the fermented dairy foods are manufactured from the milk, their composition varies more or less with the milk. In general, raw milk is composed of numerous nutritive constituents or nutrients which is a great source of proteins, lipids, amino acids, vitamins, and minerals. The average percentile composition of fermented dairy foods is illustrated in Table 12.2. The total solids are slightly more than the milk i.e., in range of 14%−18%. Fat can vary from 0.1% to 10%, which may contribute to low, medium and high fat dairy foods. Similarly, protein, lactose, lactic acid, and pH ranges as 4%−6%, 2%−3%, 0.6%−1.1%, and 3.8%−4.8% respectively. Moreover, immunoglobulin, hormones, growth factors, nucleotides, peptides, bioactive peptides, polyamines, cytokines, also contributes to the composition of raw milk, providing great nutrition to body's requirement,

substitute for magnesium, calcium, vitamin B12, vitamin B5 (pantothenic acid), selenium, and riboflavin (Bennett et al., 2013). Milk and fermented dairy foods serve as a key source of proteins, fats, and dietary energy which contributes in various proportions. On an average, 8 grams (g) of protein/capita/day, 7.3 g of fat/capita/day and, 134 Kilo calorie (Kcal) of energy/capita/day respectively, are contributed by each. However, these contributions vary significantly among different regions (Agyei et al., 2019; FAOSTAT, 2012). Because of the number of factors associated with milk composition, the chemical composition of dairy foods, specifically fermented dairy foods, also varies with variability and multiplicity of analytical techniques applied to report its composition. A proximate composition of milk constituents of cow, camel, and goat milk is reported in Table 12.1, which aids as a common source for the compositions and the main foods of various fermented dairy foods obtained from milk. Milk is considered as a primary source of protein in the human diet, providing proteins around 3.3, 3.1, and 3.4 g per 100 g of cow's, camel's and goat's milk, respectively (Bennett et al., 2013; FAO, 2013). During fermentation of milk, the proteolytic and lipolytic activities of *L. acidophilus* bacteria leads to a formation of peptides, having opiate activity, which may affects antimicrobial activity, stimulates immune response, and hyposensivity (Belitz et al., 2009; Mehta, 2015). Moreover, some *L. acidophilus* bacteria may lead to the formation free amino acids due to their proteolytic and metabolic activity (Pessione & Cirrincione, 2016). For example, milk fermentation by bacteria, like thermophilic lactic streptococci or acidophilic rods, results in release of at least four amino acids i.e., proline, valine, cysteine, and arginine along with final foods. These amino acids provide nutritive characteristics and act as an essential source of amino acids necessary for human metabolism (Akabanda et al., 2013). The following Tables 12.1 and 12.2 shows the nutritional compositions in various animals.

Apparently, milk also contains various saturated and unsaturated fatty acids constituting milk fat, with the actual concentration depending on several parameters like the cattle's origin and feed-related factors. On an average, about 70% of the milk fat is constituted by saturated fatty acids, while the remaining 30% is constituted by unsaturated fatty acids (Pereira, 2014). Out of several saturated fatty acids found in milk, palmitic, myristic, and lauric fatty acids have the characteristic of raising the cholesterol in blood. In addition, several other vital nutrients, like calcium, linoleic acid (LA), conjugated linoleic acid (CLA), probiotic bacteria, and antioxidants that impart protective and hypocholesterolemic effects are also present in milk and fermented dairy foods (Gurr, 1992; Rogeli, 2000). These foods are predominantly abundant in micronutrients, specifically calcium, and other elements namely, magnesium, selenium, phosphorus, and zinc. Milk also constitute fat soluble vitamins like, vitamins A, D, E, and K, and water soluble vitamins like, B complex vitamins, and vitamin C in raw milk (Gaucheron, 2011; Haug et al., 2007). Apart from the main constituents of the fermented dairy foods mentioned, which impart taste to milk and dairy foods, several aromas/smelling substances are also synthesized during fermentation. The non-volatile compounds provide taste, while the volatile compounds like

TABLE 12.1 Nutritional compositions of camel, goat, and cow milk (g/100 g of milk) (Bennett et al., 2013; FAO, 2013).

Cattle milk type	Water content	Total protein	Total fat	Lactose	Ash
Cow	87.8	3.3	3.3	4.7	0.7
Camel	89	3.1	3.2	4.3	0.8
Goat	87.7	3.4	3.9	4.4	0.8

TABLE 12.2 Composition of fermented dairy foods.

Attributes	Percent
Total solids	14–18
Fat	0.1–10.0
Proteins	4–6
Lactose	2–3
Lactic acid	0.6–1.1
pH	3.8–4.8

esters, aldehydes, alcohols, methyl ketones, sulfur and phenolic compounds, short- to medium-chain of free fatty acids, dicarbonyls, and lactones are responsible for both taste and aroma (Urbach, 1993, 1995, 1997). Taste and aroma together impart flavors to the fermented dairy foods, which varies greatly with the type of bacteria or microorganisms involved during its production. The following are the chemical compositions of some common fermented dairy foods; however, the values referred may vary depending upon the various factors and conditions mentioned earlier.

12.5.1 Yogurt

On an average, yogurt culture contains 9.30% ± 2.52% of total solids, 3.96% ± 0.63% of total proteins, 2.13% ± 1.28% of fat, 2.74% ± 0.21% of lactose, 0.81% ± 0.29% of ash, 0.260% ± 0.12% of acidity (lactic acid), 88.15% ± 0.02% of moisture and 5.94 ± 0.59 of pH (El Zubeir et al., 2005). However, as discussed earlier, the exact composition varies significantly depending upon several factors. An essential aromatic substance that comes in yogurt is due to the carbonyl compounds, specifically diacetyl and acetaldehyde. Additionally, along with 1-nonen-3-one, 1-octen-3-one, have also been described as a significant odorant, but with remarkably low odor (Belitz et al., 2009; Mehta, 2015).

12.5.2 Kefir

Kefir is a self-carbonated, slight alcoholic (0.5%−2.0%), and refreshing fermented yogurt beverage, having characteristic combined flavors with a mixture of carbon dioxide (1.98 g/L), lactic acid (0.5%−1.0%), acetaldehyde, acetoin, casein, and some other flavors that generate fermentation and proteolytic action of kefir grains (yeast). The composition of kefir includes 3.0% protein, 0.2% lipids, 80%−90% moisture, 6.0% sugar, and 0.7% ash (Beshkova et al., 2003; Belitz et al., 2009; Gao & Li, 2016).

12.5.3 Dahi (curd)

The major constituents of dahi are more or less similar to the raw milk with the exception of a small loss of water and acidity. The composition varies with the type of milk used for its production and also on several other factors. On an average, dahi contains, 85%−88% water, 3%−3.4% protein, 5%−8% fat, 0.7%−0.73% lactic acid, 4.2%−5.1% lactose, 0.5%−1.1% ash, 0.12%−0.15% calcium, and 0.09%−0.11% phosphorous, prepared from whole milk (Mehta, 2015; Shivashraya, 2014).

12.5.4 Sour milk

Sour milk is a thick, curdled milk, having sour taste, obtained from fermentation of milk either by LAB or by adding mesophilic microorganisms along with lactic acid, which results in the coagulation of casein at pH 4−5. Sour milk can be produced from different milk types, such as complete milk, containing at minimum 3.5% of milk fat; skim milk, having around 0.3% of fat; or even from low fat milk, having 1.5%−1.8% of fat. Sour milk contains about 0.5%−0.9% of lactic acid (Belitz et al., 2009; Mehta, 2015).

12.5.5 Buttermilk

On an average, buttermilk contains 3.0 ± 0.18 g/100 g of total protein, 1.17 ± 0.68 g/100 g of fat, 3.61 ± 0.5 g/100 g of lactose, 364 ± 250 μg/g of galactose, and 59.9 ± 100.3 μg/g of glucose. Apart from the presence of the common and most abundant lactic acid in buttermilk and other fermented dairy foods, other organic acids are also detected in buttermilk, which include citric acid, uric acid, acetic acid, a-ketoglutaric acid, pyruvic acid, orotic, and succinic acid in varied proportions. The mean of the volatile compounds, like acetaldehydes, ethanol, diacetyl, and acetoin was reported to be 0.82 ± 0.94 μg/g, 91.5 ± 85.5 μg/g, 0−7.76 μg/g, and 18.9−397.8 μg/g, respectively (Gebreselassie et al., 2016). Diacetyl imparts a buttery taste to the dairy foods.

12.5.6 Lassi

Lassi is an Indian traditional beverage, which is prepared by churning dahi/yogurt, or it is obtained as a by-product while preparing desi butter (also known as, makkan) from dahi/yogurt. The process of making lassi does not have any fixed standard method, that is why its composition varies significantly. On an average, lassi contains 95%−96% of

water, 1.10%−1.35% of protein, 0.6%−0.8% of fat, 0.38%−0.43% of ash, 1.1%−1.5% of lactose, 0.42%−0.48% of lactic acid, 0.04% of phosphorous, and 0.60% of calcium (Belitz et al., 2009; Mehta, 2015; Shivashraya, 2014).

12.5.7 Cheese

The chemical composition of cheese is varied in proportions depending on the milk composition, origin of milk, type of cheese, and various other ingredients, like cultures and enzymes involved in its manufacturing process. To obtain basic structure, texture, flavor, and composition of cheese involves having optimum pH, moisture, fat, salt, and minerals (especially calcium); proper care is taken to meet optimum requirement of microbial and enzymatic activity. The production of acid by bacterial cultures is the most vital step for this purpose. The composition and the variety of cheese inclines to differ suggestively among different lots, and even within same batch between cheese loaves and also with ageing (Mehta, 2015; Walstra et al., 2006). During the manufacturing process of cheese, a part of lactose is washed away with the whey while the rest gets fermented into various volatile and organic foods like lactic acid, acetic acid, acetaldehyde, ethanol, diacetyl and carbon dioxide. Cheese also contains essential amino acids as a source of protein. Moreover, it contains fats as a main component, which ranges between 20% and 35% of the dry mass. Out of the total milk fat; 60% are saturated fatty acids, 23.5% are monosaturated, and 4.6% are polysaturated fatty acids. Palmitic acid is the most common saturated fatty acid present in cheese, which constitutes about 26% of fat, while 8.0% is stearic acid, 9.8% is myristic acid, and the rest 0.02%−3.1% is contributed by other saturated fatty acids. On the other hand, oleic acid is the most common unsaturated fatty acid found is cheese, which constitutes around 16.5% of fat. Cheese is also rich in calcium (semi-hard and hard, ranging from 0.6% to 1.1%), magnesium, phosphorous, and zinc. Apart from these, cheese also contains several flavored compounds, which are formed as metabolic foods from sugar, fat, amino acids, and other pathways during cheese ripening process. For example, in cheddar cheese; diacetyl, acetic acid, methional, isovaleric acid, butyric acid, propanoic acid, 3-methylbutanal, methanethiol, ethyl butyrate, butanone, dimethyltrisulphide, ethyl hexanoate, and 1-octen-3-one; in Gouda cheese; diacetyl, 3-methylbutanal, butyric acid, 2-methylpropanol, butanone, pentanal, hexanal, limonene are found. Similarly, Swiss-type cheeses contains skatole, ethyl butyrate, methional, phenylethyl acetate, and some others, while Camembert cheese contains methional, 3-methylbutyrate, 2,3-butanedione, benzaldehyde, 1-octen-3-ol, γ-decalactone etc., which are found as a flavor compounds (Smit et al., 2005).

12.6 Consequences of dairy foods

The identification of specific beneficial health effects of dairy foods, including fermented dairy foods, has been reported. The high or low consumption of certain dairy foods, on one hand, has positive health benefits and nutritional impacts; on the other hand, it also has many consequences which affects both children and adult health. An amount of fitness consequences have been examined in connotation with dairy product intake, especially adiposeness, bone mineralization, dental well-being, linear development, and blood pressure (Dror & Allen, 2014; Mayorova, 2018). It is noteworthy, but not astonishing, that the two establishments, the National Hispanic Medical Association and National Medical Association, have lectured this public health issue. The 2010 DGA (Dietary Guidelines Advisory) evidence rating, showed moderate evidence that the intake of milk and milk foods is linked with an abridged risk of circulatory disease, type 2 diabetes, and lower blood pressure in adults' disease conditions affecting Hispanic Americans (HA) and African Americans (AA) at uneven tolls. The 2015 DGAC or Dietary Guidelines Advisory Committee reiterated this connotation in their technical statement. This indication makes a robust case for the presence of dairy in the diets of AA and HA. It is unspoken that fitness differences may exist amongst all ethnic and cultural marginal clusters; though, this article will focus on dairy's role in improving AA and HA health results, and plans for growing dairy feeding among these inhabitants (Brown-Riggs, 2015). The high consumption of dairy foods can also have toxicological consequences. This is because the extensive variety of dairy foods contain biogenic amines (BA). These are basic, organic, nitrogen-bearing mixes having biological activity and are mainly formed by decarboxylation of amino acids; they can accumulate in the body in high amounts. The consumption of large amounts of some dairy foods, e.g., some variety of cheese, having large amount of BA, can result in the accumulation of BA in the body; hence, causing toxicological effects. Roughly, added 1000 mg of BA has been noticed per kilogram of cheese. Though there is no exact law concerning the BA contented in dairy diets, it is usually expected that they should not be permitted to collect. Better information of the influences consisting in the amalgamation and gathering of BA should lead to a decrease in their occurrence in foods. Moreover, milk and dairy foods are rich origin of calcium. The high of intake dairy foods results in high consumption of calcium, which may have a subsequent impact on health and chronic diseases in children and adults.

Consequently, excess iodine consumption from a source of some dairy foods, can result in thyroid dysfunction in certain susceptible individuals, but is generally well tolerated in most people (Leung & Braverman, 2014). The part of fermented milk foods on humanoid well-being has been the topic of widespread study, consisting of epidemiologic, experimental, and scientific lessons (Savaiano & Hutkins, 2021). The rising marketplace of medicines, dietary additions, and chiefly foods created on living probiotic bacterium, is indication of their adequate preventive and, if less clear, beneficial properties. But it is presently not possible to exactly regulate the optimal amount of microorganisms essential for valuable probiotic properties on social fitness; in many cases, precise statistics are absent on probiotic effect mechanisms and marks. Results are that the optimistic effects of live probiotic bacteria may be short-term, indeterminate, or even absent in case of long use. The nonappearance of clear health-promoting consequences of given probiotics on the base of live animals is typically clarified by low attention of physically active bacterial mixes where they are applied (Reid et al., 2011). Importantly, a growing amount of food manufacturers are beginning to produce functional foods, described as food with additional health benefits in addition to its existing nutrient content (Rincon-Leon, 2003), one of which is fermented food and dairy foods.

12.7 Physico-chemical characteristics of fermented dairy foods

Milk and fermented dairy foods possess considerable variation in physical as well as chemical characteristics depending upon the type and variety of milk used for fermentation, environmental and fermentation conditions, breed, lactation period of cattle, and many other factors. This means that there exists distinct variation in the physico-chemical features amid cow, camel, goat, and sheep. The physical characteristics (color, texture, moisture content, pH, specific gravity, titratable acidity i.e., amount of lactic acid, viscosity, conductivity), and the chemical characteristics such as the amount of total protein, total fat, lactose, casein, mineral elements (Na, Mg, Ca, K), and ash, together are the important parameters to study the physico-chemical characteristics of fermented dairy foods (Dobrzanski et al., 2005). There occurs slight variations in color among different fermented dairy foods, ranging from pale white to a brownish color. During the fermentation process, a remarkable change is observed in the pH of fermented dairy foods. The fermented dairy foods (e.g., yogurt, kefir, dahi, etc.) are usually determined by digital pH meter, and have lower pH than raw milk due to the lactic acid formation, hence, increasing the acidity and lowering down the pH. This decrease in pH is less important for the yogurt manufactured with ewe's milk with an average of 5.56 compared to 4.34 for the other samples of milk. This result varies in the composition of each kind of milk described. During the manufacturing process of fermented dairy foods, the pH values of milk samples start to decrease from the time it was inoculated with bacterial cultures to the time when it was manufactured, which ranged from 6.7 to 4.34. The differences in pH of fermented dairy foods can be pretentious by several influences like quantity and type of first course culture and whey used, pH maintained at the time of fermentation process, activity of bacteria, amount of different compositions, and time and environment of storage (Thamer & Penna, 2006). Additionally, the texture, viscosity, and specific gravity varies depending upon the moisture content of different fermented dairy foods, as can be understood by the cheese variety classified, depending on the moisture content in Fig. 12.1. Fermented dairy foods may have lower total protein content compared to raw milk because of the fact that starter cultures and whey are added to the raw milk for fermentation (more detail is discussed in chemical composition of fermented dairy foods) (Brazil, 2005). Similarly, fat content varies significantly among different fermented dairy foods, cheese having the highest total fat amongst all others. evaluated the best chemical features of kefir, a fermented dairy produce prepared from goat's milk, cultured with 7% (w/v) kefir grains, which was gestated for 24 hours. The percentage values of pH, titratable acidity, ethanol, and lactose content evaluated was 4.37%, 0.76%, 0.91%, and 4.23%, respectively. Zubeir et al. (2012) evaluated the processing properties and chemical characteristics of yogurt produced from different milks and concluded that, ovine milk had the highest viscosity, subsequently followed by caprine, bovine, and camel milks.

12.8 Role of microbiological characteristics in fermented dairy foods

Microbiological characteristics possess a notable part in fermented buttery foods. Specific metabolites produced in the dissolving process by LAB inhibits the growth of undesirable microorganisms. Therefore, the bacteriological superiority of milk, from the fact of milking from a fit animal to fermentation, is hypothetically predictable to be harmless for humanoid feeding. Though, when it is concealed from the bag and proceeded for processing and storage, milk can effortlessly be dirtied by decay microbes and food-borne pathogens from numerous foundations counting bodily feces, mud, midair, food, liquid, apparatus, animal skins and individuals. Consequently, the occurrence of infective and decay bacteria in milk and dairy goods is predisposed through a great quantity of aspects and its amalgamations. These issues

may contain fitness position of the dairy cow, sanitation level in the dairy ranch atmosphere, milking and pre-storage circumstances, accessible storing amenities and skills, field organization performance, topographical position and term (Muehlhoff et al., 2013; Oliver et al., 2005). In adding to bacteriological dangers, milk and dairy foodstuffs can also comprise biochemical threats and pollutants, mostly from the atmosphere, animal feedstuffs, animal farming, and manufacturing practices. So care and manufacture are fundamentally connected in the dairy nutriment manacle; from overseeing management and dispensation to ingesting. Consequently, in the direction to minimalize the security dangers accompanied with milk and dairy foodstuffs, there is the necessity for a unremitting scheme of defensive procedures commencing with the security of animal fodder, with decent agricultural performance and on-farm panels, to virtuous trade and cleanliness, customers protection alertness, and correct claim of food safety administration arrangements throughout the dairy process (Kenny, 2013). The microorganisms isolated from fermented dairy foods are varied, counting aerophilic mesophiles, LAB, yeasts, and enterobacteria.In this technique, the fermentation procedure can be meticulous, thus adding to the superiority of the creation (Akabanda et al., 2013). From a good physically healthy animal, raw milk is likely to not harbor microbes at the time of gathering. Though, this is rarely the occasion. Usually, infective bacteria can pollute fresh milk primarily, endogenic adulteration happens when milk is polluted by a straight transmission of microbes from the plasma (complete contamination) of an diseased animal into the liquid, or via an contagion in the udder. The subsequent resources by which new milk can be polluted is recognized as exogenic adulteration, where liquid is polluted throughout or afterwards, gathered by animal feces, the external of the udder and teats, the casing, and additional ecological causes (Verraes et al., 2015).

12.9 Conclusion and future perspective

Milk is considered as one of the sources of a nearly complete nutritious diet required for human health. The variety of fermented dairy foods like yogurt, kefir, dahi (curd), sour milk, buttermilk, and different varieties of cheese, are manufactured after fermenting the raw milk with different starter cultures or lactic acid microbes and microorganism at suitable temperature and condition. The introduction of starter culture and certain bacteria for fermentation not only increases the shelf-life of produced fermented dairy foods, but also are well known to suppress the activity of undesirable bacteria and other disease-causing microbes in the human intestine. Reliant on the sort of starter culture or microorganism and configuration of lactic acid involved, the flavor, texture, aroma, and composition of fermented dairy foods varies to a significant extent. This greatly affects the physico-chemical characteristics of fermented buttery foodstuffs. The pH of fermented dairy foodstuffs are usually found lower than the raw milk due to the involvement of LAB, hence increasing the titratable acidity. Total protein content, lactose, fat, casein, vitamins, and minerals, contribute to the nutritional significance of milk and fermented dairy foods. The chemical structure of milk and fermented dairy foods differs greatly, depending on several factors like type of cattle, breed, lactation state/stage, seasonal variation, kind of milk utilized, method of preparing dairy foods, and other environmental conditions. Therefore, these factors have a major part in clearly defining the composition of milk and other dairy goods including fermented dairy foods.

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Chapter 13

Advancement in cheese production technology

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13.1 Introduction

Over the past 30 years, milk production around worldwide was 843 million tons. In recent decades, more than 150 million households across the globe are primarily engaged in milk production, and the developing countries contribute a significant share. India has been the largest contributor across the world holding 22% of the global milk production. In developing countries, milk is produced mostly by the small-holders that provide a livelihood, food security, and, importantly, cash income. It has been estimated that over 6 billion people worldwide consume milk and their products in which cheese is the most consumed milk product. The cheese has been regarded as a functional food product due to its composition and nature of microbes used that confer health benefits. The demand significantly increases every year as per the likelihood of the consumers (FAO, 2020).

Cheese is a fermented milk-based dairy product; there are over 200 types of cheeses worldwide. Cheddar, feta, Domiati, Gouda, Roquefort, Camembert, Mozzarella, and Parmesan are some common varieties of cheese found across the globe. Such varieties of milk come from different dairy species like a cow, buffalo, goat. There are coagulating agents used like rennet, pepsin, that vary the texture and moisture content, matured, or freshly processed cheese, and microbiota of the cheese (Khattab et al., 2019). The cheeses are broadly classified based on the moisture content into soft, semisoft, and hard cheese; their consumption or acceptability features depend on flavor and aroma types, organoleptic properties. Such properties are governed by various intrinsic factors of cheese, such as free fatty acids, volatile organic compounds (VOCs), amines, ketones, free amino acids (FAAs), phenolic, aldehydic, lactonic, alcoholic, and sulfur compounds (Tilocca et al., 2020). However, the most important role for cheese production is in the hands of milk microbiota. The milk to cheese process is very sophisticated, which has inherent dynamic microbiological and biochemical roles. The milk has rich nutrients; the microbiota includes *Pediococcus* sp., *Leuconostoc* sp., *Streptococcus* sp., *Lactococcus* sp., that act as a primary culture for the breakdown of nutrients such as carbohydrates and proteins into simpler forms. These forms are utilized by a secondary culture often called secondary lactic acid bacteria (LAB), which includes *L. lactis*, *L. helveticus*, *S. thermophiles*, and *L. delbrueckii*, that contribute to the final product (Gobbetti et al., 2018). The abiotic factors like temperature, humidity, and biotic factors like pH, moisture content, water activity, etc., play an important role in maintaining optimum growth of the microorganisms beneficial for cheese production. The microbial culture also contributes to the most important process in cheese production, known as ripening. The ripening process involves various biochemical influx from diverse microbiological sources. In general, the ripening of the cheese is due to biochemical events that involve various enzymatic reactions. There are mainly three processes that act on the ripening of the cheese: the residual lactose, lactate, citrate metabolism; proteolysis of the residual proteins; and the lipolysis of the residual fats or lipids (Khattab et al., 2019). The degree of cheese ripening affects the organoleptic properties and their market values. For the market value cheese production, there are highly sophisticated techniques or methodologies introduced and being practiced for increasing the quality and organoleptic values.

There has been a significant increase and advancement in the field of cheese manufacturing at every step of the process. Three major innovations have revolutionized some cheese-making sectors such as refrigeration, commercial starter and nonstarter cultures, and pasteurized milk. The standardization of the milk to concentrate essential elements in milk for the process, like more total solid content, is a necessary and important step to increase the quality of the cheese.

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There are various newly developed techniques for achieving this like ultrafiltration (UF), membrane filtration, microfiltration (MF), diafiltration, etc. The study of the genetics of starter cultures offers more reliability of the fermentation process with the development of feasible automation. Likewise, the gene manipulation techniques, like recombinant technology, along with gene transfer have been successfully applied to existing microbial strain earlier for the generation of highly efficient microbial strains. They offer more economic importance, high acid formation, bacteriophage resistant, bacteriocin production to inhibit pathogens, and control of the flavor development (Beresford et al., 2001). For example, the recombinant microbial strain with animal-produced chymosin has been approved and used by many producers. There has been a significant increase in cheese plants depending upon the specialty of the cheese manufactured. Such an increase was expected because of the people's choice of cheese from a variety of cow, sheep, and goat milk products. New advancements like manufacturing cheese using different sources of milk and even blends of different milk in creating varieties have been achieved in various parts of the world (Johnson & Lucey, 2006). The by-product whey, produced during the cheese-making or milk coagulation process, has been utilized in the generation of ecologically important fuel, as supplements in various food products, and in the bioremediation process. There are various emerging and attractive techniques developed for successful utilizing the waste whey (Li et al., 2020). There are many scientific investments giving careful study of the fundamental of cheese, making for more emphasis on the nutritional aspects. Also, the FDA (Food and Drug Administration) has enforced certain guidelines under the Food Safety and Modernization Act to achieve cheese production with minimal contamination.

13.2 Process of cheese production

13.2.1 Standardization of milk

The milk quality affects the characteristics of cheese production. The milk composition is affected primarily by safety from pathogens like *Salmonella* sp., *Listeria* sp., *Campylobacter* sp., *Escherichia coli*, *Mycobacterium bovis*, and *Brucella* sp. that poses a threat to human health. To be free of adulteration, there should be knowledge of chemical properties like proteins (especially casein), fats, minerals, water; and physical properties like moisture content of nonfat molecules and structure, viscosity, and density of the fats globules and how they affect the quality of milk. The most striking feature that diversifies the varieties of cheese is the protein to fat ratio (PFR) of the milk. PFR broadly classifies cheese into low-fat and full-fat. This has a high influence on cheese composition, yield, rheology, flavor, and organoleptic features (Bojanić-Rašović et al., 2013; Guinee et al., 2007). However, the composition of milk varies within the lactating animals, nutrition, and seasons (Guinee et al., 2006). Hence, standardization is a necessary step to maintain factors like protein (casein), fat, and pH of the milk, as these factors affect the gelation rate and later the cheese characteristics.

The most common technique used for standardization of milk is membrane filtration techniques; there are many types such as reverse osmosis (RO), UF, nanofiltration (NF), and MF. These filtration types perform similar functions of removal of pathogenic bacteria and their spores, concentrate whey, mineralization of whey, fractionation and concentration of milk proteins, and thus increase the cheese yield. The function solely depends on the porosity of the filtration technique used that retains or permeates the particular component. The most common and preferred filtration technique used in the cheese plant is UF. The principle of UF is when the milk or any emulsion passes through a semipermeable membrane that retains a high concentration of fat, proteins, and insoluble salts whereby, permeates most of the water, soluble salts, and nonprotein nitrogenous compounds (Kosikowski, 1974; Lauzin et al., 2020). Depending on the concentrate of the total solids suspended in the milk (also known as concentration factor), the UF is of three forms: (1) low concentration factor UF standardizes the milk with a proper concentrate of total solids followed by cheese production and whey separation using a conventional method; (2) medium concentration factor UF concentrates the total solids in the milk followed by cheese production through acidification and gelation process without whey expulsion; and (3) high concentration factor UF is similar to that of medium concentration factor UF, however, utilizes specialized equipment for curd formation and finally to achieve the final product, cheese (Soodam & Guinee, 2018) (Fig. 13.1).

13.2.2 Pasteurization of milk

Quick heating of milk at 160 °F to 180 °F tends to positively improve the quality of the milk and hence the cheese manufactured. The pasteurization of milk is a necessary step because raw milk carries a great risk potential for human health. The unpasteurized milk has many undesirable compounds like mycotoxins, drug residues or antibiotics, hormones, pollutants from industries, and pesticides. The milk also has many zoonotic pathogens as well as contaminants

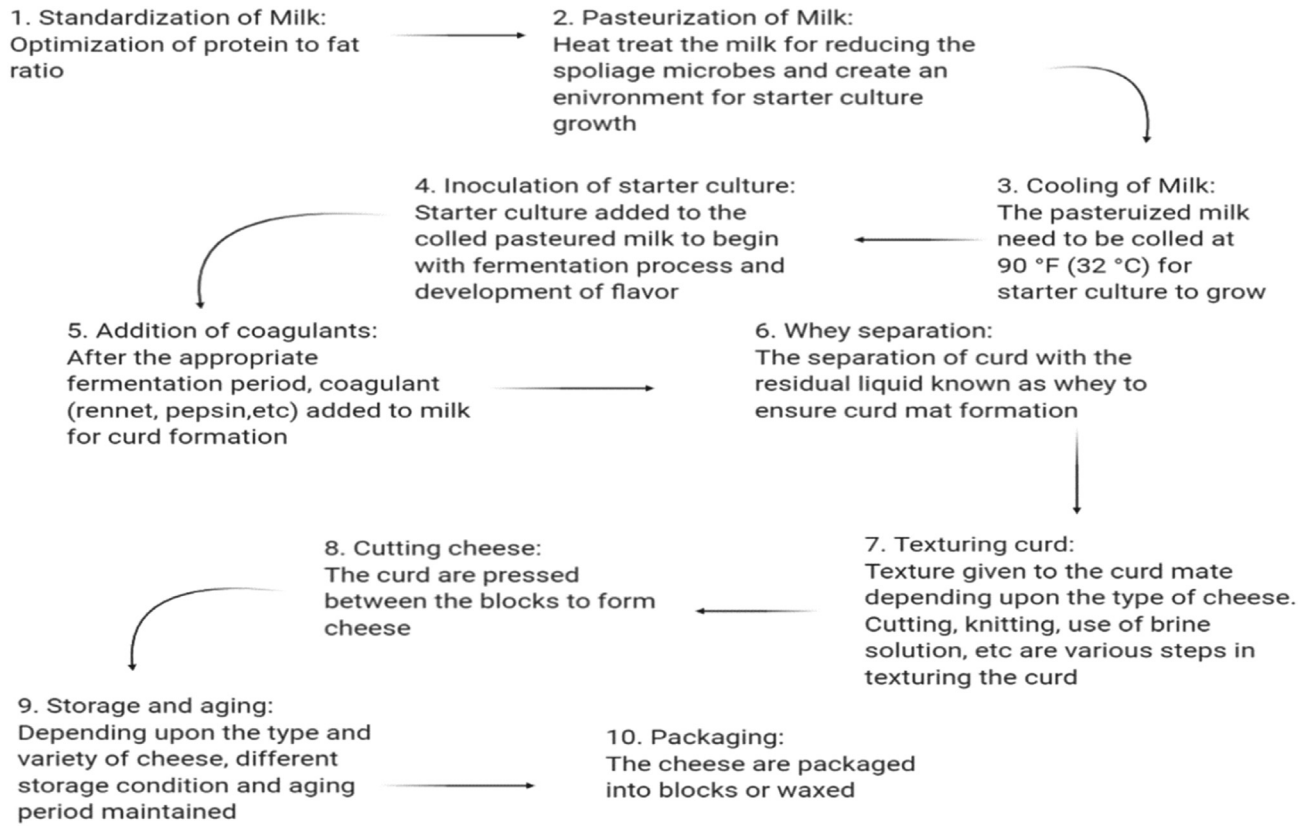

FIGURE 13.1 Summarized cheese production process.

from storage places. These microorganisms include *Listeria monocytogenes*, cytotoxin-producing *E. coli*, *Salmonella*, *Campylobacter*, *Brucella*, etc. All these compounds and pathogens widely affect the milk composition, physio-chemical, nutritive, and organoleptic or sensory characteristics of the cheese manufactured. The prescribed heating condition or the standardization of the pasteurization process depends upon the time and temperature combination. As the uncontrolled pasteurization process could coagulate, whey proteins interfere with the casein-formed micelles and lower the efficiency of the coagulation process (Phillips, 1928; Singh & Waungana, 2001; Tadjine et al., 2020). The most common type of pasteurization technique used in the cheese plant is HTST (high-temperature short time) pasteurizing method. The name itself gives an idea of its principle that the milk is heated at high temperature, here 72°C is commonly used, for a very short period of time say for 15 seconds. Once the pasteurization is over, the milk is cooled and prepared for various biochemical reactions using microorganisms.

13.2.3 Starter and adjunct/secondary culture

After the pasteurization step, the milk needs to be cooled down to a temperature required by the starter culture to grow. The starter cultures are the microbial consortia already present in the milk as its microbiota, or they could be added into the milk. The primary function of the starter culture is to start the acidification process, that is, the formation of lactic acid, which is the primary acid present in the cheese. Lactic acid production lowers the pH from 6.6 to 4.2 which leads to the curding of the milk (aka gel syneresis) and, hence, increases the shelf life of the milk. The curding of the milk is followed by the whey separation and compaction of the curd. The additional function of lactic acid production behaves as an antimicrobial agent for the inhibition of the defect causing or contaminant microbes. The primarily used starter cultures or microbes found in the milk are LAB (*Lactobacillus lactis* and *Streptococcus thermophilus*) because of the microbial status as GRAS (Generally Regarded As Safe by FDA) or QPS (Qualified Presumption of Safety by European Food Security Agency). These microbes perform oxidation and reduction processes that breakdown the complex carbohydrates to organic acids (in this case lactic acid), alcohol, and carbon dioxide via substrate-level phosphorylation coupled with fermentation. These processes affect the final cheese quality and characteristics. The pH lowering

effect is seen due to the fact that the ATP generation through fermentation is coupled with and regulate proton motive force (Bassi et al., 2015; Tilocca et al., 2020).

The secondary culture or nonstarter culture has a function in the ripening of the cheese to achieve organoleptic and sensory characteristics. The microbial groups involved in secondary culture are mesophilic facultative and obligate heterofermentative lactobacilli, also thermophilic strains of LAB. *Lactococci, Pediococci, Enterococci*, and *Leuconostoc* are the main genus of the secondary cultures. The lactic acid produced by the primary culture behaves as a substrate for secondary cultures. The residual sugars are broken down by these cultures to form pyruvate, and enter fermentation to produce aromatic compounds such as diacetyl, acetoin, and butanediol (Gobbetti et al., 2015; Steele et al., 2013; Zuljan et al., 2016). Soon after the various biochemical reactions are achieved, the milk undergoes the coagulation step for the formation of curd.

13.2.4 Coagulant used

Coagulation is the process of protein coagulation for processing milk to cheese, an important step. The process can be referred to as an indirect method for the preservation of milk. The coagulation occurs with an important milk protein called casein. The casein molecule is composed of five fractions: alpha-S1 casein, alpha-S2 casein, beta-casein, gamma casein, and k-casein. The chemistry of the coagulation states that, the casein molecule is broken by the action of any coagulant agent into five different fractions and the fractions combine with the calcium ions present in the milk to form a mass of curd called coagulum. The alpha-S1 casein, alpha-S2 casein, gamma casein, and k-casein have a negative charge and are hydrophobic, which tends to be present inside the coagulum; whereas, the hydrophilic beta-casein will face the outside (Kethireddipalli & Hill, 2015).

There are two types of coagulation processes: (1) Acid/lactic coagulation, which occurs during the starter culture fermentation process that forms lactic acid and lowers the pH. The lowering of the pH and the optimum temperature favors the breakdown of the casein proteins into different fractions and interaction between them leads to the formation of the curd. (2) Enzymatic coagulation uses enzyme chymosin from rennet that breaks the k-casein in the casein proteins to form coagulum using calcium ions. The enzyme-based coagulation is more efficient and widely used in the cheese plant due to the large coagulum formation and also expels the liquid whey with the small amount of lactose, salt, and fat trapped in the mass more efficiently. The enzyme chymosin present in rennet has three sources: animal, vegetable, and microbial. These different sources of rennet are used, depending upon the type and varieties of the cheese to be manufactured. After the coagulum/curd formation, the residual liquid whey is separated out. Furthermore, the processing of coagulum is done to achieve maximum separation of whey from the mass of casein micelle. This step varies with the type of cheese and curd that is pressed within the blocks to separate out the whey.

13.2.5 Texturing and cutting

One can define texture as a sensory property with varying multiparameter attributes derived from the cheese structure. In other words, the texture of any material like food should be analyzed through the human touch perception and visualized through the food physical characteristics, not just by the tenderness or chewiness of the food. The texture of the cheese depends on the ripening process. It has many determining factors that can affect the texture like manufacturing and procedural variety, composition, and biochemical changes that occur during the ripening process (Enab et al., 2012). The cheese is also processed using a brine solution in order to slow down the activity of the starter culture for enhancement of the ripening process. The brine solution tends to decrease the moisture content and thus increase the yield. Also, the brine solution provides a smooth texture to the cheese and reduces the hardness. A 4%−6% brine solution has been used for this purpose (Farkye, 2004). After analyzing the texture, the cheese is cut into the blocks depending upon the packaging strategies.

13.2.6 Storage and packaging

In order to reduce the deterioration of the quality of cheese, the storage and packaging steps need to be handled very cautiously. As the cheese is the most frequently used item in the food products, there is a need to focus on the storage facilities and packaging including establishing a link to the dealers and the consumers. The temperature-based storage allows the cheese to further ripen and age in order to develop more sensory characteristics. The cheese plant follows the Hazard Analysis Critical Control Point principles to ensure the safety of the cheese for the consumers. The ripening

of the cheese contributes to the reduction of any contaminants during storage (Bishop & Smukowski, 2006; Zehren, 1984).

13.3 Factors affecting the quality of cheese

There are various factors that influence the quality of the cheese. Each factor is contributed towards the texture, sensory, and organoleptic parameters of the cheese.

13.3.1 Milk and related factors

At the precheese production stage, all the factors involved at this stage revolve around the milk. The common milk components are proteins (casein and whey), fats, carbohydrates (lactose), soluble and insoluble minerals and vitamins, and water. The various factors that affect the milk and its cheese potential are the composition of milk, casein biology, microbiota, and storage of milk.

13.3.1.1 Composition of milk

The fat to protein ratio primarily influences the composition of milk. The concentration of fats in milk varies with feeds given to the ruminants. Although, there is no change in protein concentration upon feed change. The protein part in the milk changes due to the genetic variation among the species of ruminants (Amenu & Deeth, 2007). For instance, the pasture-derived milk has a higher concentration of fats than proteins; whereas, the reared-derived milk, where the animals feed on a particular feed given by their owners, yields low levels of both fat and proteins. The type of season has a varied composition of milk from ruminants. It has been proven that during winters, milk has a high level of both mono-unsaturated fatty acid and poly-unsaturated fatty acid than during summers (Romanzin et al., 2013). The proteins and fats concentration in the milk affects the potential cheese production. The recovery of protein and fats in the curd or cheese upon milk-processing is the key aspect of the high cheese yield. So estimation of the protein and fats in the milk and cheese gives a clear idea about how much of the components have been retained in the cheese and expelled in whey separation. For instance, goat milk is rich in a higher amount of fats and proteins which increases the recovery efficiency of the other nutrient components during the curd formation, ultimately improving the potential cheese produce (Pazzola et al., 2019).

13.3.1.2 Casein variants or fractions

As we are aware of the milk protein, casein, has five genetic variants or fractions: alpha-S1 casein, alpha-S2 casein, beta-casein, gamma casein, and k-casein. These variants are highly associated with milk yield and composition that affect the cheese in terms of curd formation, gelation ability, and retention of moisture. The primary function of the casein fraction is to form casein micelle and helps in syneresis. However, the size of the micelle formation, concentration, and association of calcium phosphate in the curd gelation and the casein proportion in the milk makes a great difference on the cheese production. Among the five fraction of the casein, k-casein has shown a major role in the gelation of milk to curd. The increased fraction of k-casein positively correlates with an increase in curd formation. Although, beta-casein has a negative effect that masks k-casein during the cleavage process of the coagulation (Cipolat-Gotet et al., 2018). This decreases the size of the casein micelle which results in poor gelation and hence, poor cheese production. Casein fraction, particularly, k-casein also affects the time period of gelation. A higher concentration of k-casein of BB genotype showed a shorter time period for the gelation process with maximum fat and proteins in the curd than in whey (Alipanah & Kalashnikova, 2007). The relative content of the k-casein of the BB phenotype has proven to improve the milk coagulation process of many kinds of cheese like cheddar, Gouda, and mozzarella. The degree of glycosylation also affects the involvement of k-casein in the coagulation process, as glycosylation improves the milk coagulation step (Bonfatti et al., 2011; Robitaille et al., 1993). The alpha casein fractions also play an important role in casein micelle formation as their presence has a positive effect.

13.3.1.3 Microbiota of milk

Understanding the microbiome in milk is an important factor to ensure some consequences brought by such microbes on the finished cheese product. The microbiome in milk widely affects the texture, organoleptic, and perishable characteristics of the cheese in order for its safe consumption to the consumers. It has also been documented in many kinds of literature that microbiome contributes to additional quality. The synthesis of different industrially important fatty acids

that enhance the organoleptic properties of the cheeses is one aspect. Milk is thought to be a vector for the transmission of microbes from being in raw material to the finished product. As a matter of fact, the milk microbiome itself has various factors like pH, water activity, relative humidity, and temperature that affect the growth of the microbes. However, there are other critical various possible factors that can adversely affect the microbial communities in the milk through the change in microbial consortia in the udder. Such an effect may be due to animal husbandry practices that involve reared feeding, milking, and the cleanliness of the surrounding. However, the storage conditions depending on the place, air, and dust also affect the microbial communities. The milk microbiota is also influenced by the environment-based pasture foraging (grassland, silage, hay, etc.). This also affects the udder microbiome. The milk microbiome consists of a high number of bacterial genera followed by molds and yeasts. Particularly in bacteria the diverse group present are LAB, *Actinobacteria, Staphylococcus, Streptococcus* genera, and some gram-negative genera (Milani et al., 2019; Tilocca et al., 2020). Some of the microbes in the milk are regarded as pathogens, and, if left unattended during the process of cheese production, can affect the cheese quality during ripening due to the fact that the groups have a wide temperature for their growth. From psychrophilic to thermophilic, the pathogens can withstand cold storage temperature to high-temperature pasteurization.

13.3.1.4 Storage of milk

Once the milking has been done, the milk needs to be stored in a cool place to avoid spoilage before being taken to the cheese plant or in case there is a delay in transportation. Different literature cites that the on-farm storage for the milk needs to be maintained below 8°C. However, the long delay for the milk to be transported increases the expenditure for the animal husbandry workers. Thus, they tend to store milk slightly above the optimum temperature in order to save their expenses, and in doing so, the chances of milk spoilage increase. Milk spoilage has been observed due to prominent psychrotrophic microbes. The most common genera are *Listeria monocytogenes* and *Pseudomonas*. These microbes, upon reaching the stationary phase of the cell cycle, produce enzymes like lipase and heat-stable proteinases. Such enzymes bring out the proteolytic and lipolytic degradation of the milk components like casein and lipid or fat into smaller and simpler forms. Thus, these affect milk-processing during the pasteurization, where the milk tends to coagulate and impairs the coagulation process (De Moura Maciel et al., 2015; O'Connell et al., 2017). Also upon transferring stored milk to the cheese plant, additional storage at the plant during the in-between cleaning process of the plant causes the psychrotrophic to grow in full swing and thus impairs the whole cheese process. This causes the loss of economic and energy expenditure which can be tolerated in industries. Hicks et al. studied the effect of low temperature on the milk storage for cheese production. They inferred that cheese quality and psychotropics have an inverse relation. In other words, the higher growth of psychrotrophics decreases the cheese quality due to the formation of gassy, unclean, and bitter-flavored cheese (Hicks et al., 1986).

13.3.2 Factors during the process

The various factors included here are at the cheese manufacture level which includes standardization, pasteurization, coagulation, whey separation, addition of brine solution, texture and cutting, storage, and packaging.

13.3.2.1 Standardization of milk

The standardization of milk is an important aspect to increase milk constituents like fat and proteins in order to achieve high economic cheese. The addition of powdered or liquid skim milk or protein concentrates and the removal of fat has been a suitable option for doing so, owing to its advantages and disadvantages. The standardization of milk is done, as discussed earlier, depending on the type of cheese to be manufactured. However, the problem associated with the addition of skim milk powder has been documented in the report of Rehman et al. They inferred that the addition of skim milk powder or concentrate increases the residual lactose content in milk that favors high lactic acid formation which affects the ripening and sensory characteristics of the final cheese product upon storage (Rehman et al., 2003). The increase in the fat content of milk positively correlates with the decrease in moisture content in the final cheese (Fenelon and Guinee, 1999). The addition of lactose-based skim milk powder also affects the hardness of the cheese. In one report of Moynihan et al., the significant increase in lactose to casein content, that is, HLC (high lactose to casein ratio) during the standardization of milk showed a hard texture to the final mozzarella cheese product than medium lactose to casein and low lactose to casein ratio-based mozzarella cheese. The casein and fat content in each case was kept constant (Moynihan et al., 2016).

13.3.2.2 Pasteurization

The pasteurization of milk is done to ensure the safety of milk to be processed for cheese production by eliminating the milk-borne pathogens. Although, the preferences for raw milk cheese and pasteurized milk cheese among the consumers go hand-in-hand. However, the preference for raw milk cheese has been adapted more for the consumers (Colonna et al., 2011). The possible reason for this likeliness lies in the sensory and organoleptic characteristics of the cheese. The pasteurization offers advantages such as enhanced and uniform cheese quality and yield But it constricted to only a few industries because of low flavor development and high cost and availability of the equipment. During the pasteurization process, some of the milk components, such as heat-labile proteins, certain mesophiles, and heat-labile indigenous enzymes, are eliminated which could otherwise contribute to more flavors to the pasteurized milk cheese. The pasteurization of milk has a negative effect on the fat content of milk (Tadjine et al., 2020). The prolonged use of pasteurization equipment led to the development of biofilms. The biofilm develops from bacterial adherence to the walls of regenerative heaters of the pasteurization equipment, which secrete exopolysaccharides to develop biofilm that adheres more bacteria. This leads to postpasteurization contamination of the milk.

13.3.2.3 Coagulant used

The yield and quantity of the cheese depends upon the type of coagulant used. As discussed earlier, there are various sources of coagulant that affect cheese production. In general, coagulation can be defined as the degree of casein micelle formation. The most common coagulant used in the cheese industry is rennet, and the activity of rennet depends on the pH under which the process is held. Earlier, the use of rennet was not feasible due to high cost and certain religious facts (Liburdi et al., 2019). Also, the pH has a significant effect on coagulation that affects the final cheese product. For instance, the use of rennet for milk coagulation under low pH produces cheese of high moisture with a greater melting effect. However, lowering the pH below five causes numerous small casein aggregations with low interaction which causes loose and brittle curd formation. This also affects the hardness of the cheese and becomes less palatable because of less chewiness (Johnson et al., 2001). There are certain plant-derived coagulants used for the production of cheese with some benefits as well as a negative impact; for example, the use of fig latex showed a decreased syneresis rate, yield, and quantity, and affects the sensorial properties of the cheese (Bornaz et al., 2010). So, plant-based coagulants are seldom in cheese industries.

13.3.3 Postcheese production factor

13.3.3.1 Storage condition

The storage conditions and packaging methods of the cheese are associated with an increased shelf life of the product and safe consumption to the consumers. The storage temperature and packaging affect the cheese texture, quality, and sensorial parameters. The improper storage of the cheese results in excessive ripening that can be home for many psychrotrophic pathogens and degrade the quality of the cheese. Packaging under improper atmospheric carbon dioxide also affects the sensorial parameters of the cheese (Jalilzadeh et al., 2015).

13.4 Advancement in the cheese process

Over the past two decades, cheese consumption across the globe has increased and so as the production. The processing of large amount of milk into cheese has encouraged industrialists to change or update the process for its maximum cheese potential with less accumulation of whey waste. Many advancements have been done through changes in milk standardization techniques; for example, the use of computer software for monitoring each step of the cheese production. There have been significant changes in the protocols to minimize the manufacturing time through the use of recombinant techniques along with genomics and proteomics for the choice of microbial-based coagulants, starter, and nonstarter cultures. The usage of adjuncts in enhancing or adding more flavoring components to the cheese. The following context deliberately focuses on recent advancements achieved in cheese technology.

13.4.1 Trend of milk standardization

Due to the factors described earlier, membrane filtration techniques are widely used in the standardization of milk. The current scenario has led a revolution in the membrane process in dairy industries. The membrane filtration has a wide spectrum of techniques such as MF, UF, NF, and RO commonly used in the cheese factories, each having their own specificity.

13.4.2 A microfiltration

The MF of different membrane pore size is utilized for the separation of molecules having 200,000 Da. The process effectively separates or removes microbial cells, somatic cells, casein, fat globules, whey, and suspended particles (Dhineshkumar et al., 2015; Nelson & Barbano, 2005).

13.4.3 Ultrafiltration

The separation of molecules having the molecular size in the range between 1000 and 100,000 Da. The process is commonly used for the separation of proteins, fats, and colloidal salts (Dhineshkumar et al., 2015).

13.4.4 Nanofiltration

The separation of molecules having the size in the range of 200−2000 Da is achieved using this process. It has applications in partial demineralization (Dhineshkumar et al., 2015).

13.4.5 Reverse osmosis

It is also known as hyperfiltration, where water is passed through the membrane and works against the osmotic pressure. It retains all the solutes including lactose, salt, colloidal minerals, as well as undissociated minerals that have a size range of 150 Da or less (Dhineshkumar et al., 2015).

Generically, the aim of all the techniques emphasizes the molecular separation of the desired components upon applying pressure (transmembrane pressure). Such a pressure-driven process passes through various membranes of choice that results in two parts: retentate/concentrate and the filtrate. The choice of product could be present in both parts. The UF technique helped in milk concentration through maintaining buffer abilities and the acidifying role of starter culture during the production of cheddar cheese (Boivin-Piché et al., 2016).

13.4.6 Trend of pasteurization of milk

The need for pasteurization of milk started back in 1914. Till then, the raw milk was delivered in cans that had a capacity of only a few hundred to thousands kg to the cheese plants. Low-temperature pasteurization or vat pasteurization technique was used which cooled milk under cold spring water that had a temperature around 12°C. However, the milk was spoiled at a much higher rate of about 1 million bacteria per mL of raw milk, and homogenization was not achieved. These high numbers did not favor production until the number has been bought to a safe level. Another problem with poor storage and delivery of milk was rancidity. This phenomenon was caused by milking from ill animals that carry mastitis in milk. However, during the developing years, the delivery in cans was replaced by specialized refrigerated milk trucks that would carry around 23,000 kg of milk at one time. The new animal husbandry practices achieved by workers and industries like cleaning, sanitation, and cooling techniques have now decreased the contamination of the milk to a safe level of 20,000 CFU bacteria per mL of the milk. Pasteurization has the biggest advantage of controlling acidification in order to control the quality. During pasteurization, microbial contaminants are killed that would otherwise ferment lactose to give undesirable flavor to the cheese. Through killing such contaminants, the desirable use of starter culture has increased. It would offer control of the acidification and improve the efficiency of the whole production in fixed time scheduled production. The low-temperature pasteurization was replaced by HTST due to the inability of the former to process huge tons of milk. HTST was introduced in 1933 and is still the most widely utilized pasteurization equipment, due to its high volume throughput, which necessary as the industries receive tons million of milk to get processed (Holsinger et al., 1997). The FDA and United States Public Health Service administered an ordinance called Grade A Pasteurized Milk Ordinance that defines practices regarding milking and the use of pasteurization for grade A milk products, including cheese. According to which the milk pasteurization through HTST requires the processing of milk at a temperature of 72°C for a 15 seconds period (Ranieri et al., 2009).

13.4.7 Trend of milk coagulants

The use of calf rennet for milk coagulation was at its high potential during 1961, but soon reduced due to the high cost of purchase, reduction in supply, and vegetarianism. The current trend and research have focused on applying natural products as a milk coagulant. The plant-based coagulants have gained faster popularity due to immense benefits like

improved rheology, sensorial properties (texture, taste, color), and high enzymatic and proteolytic activity. Also, the extraction of coagulant compounds from plants is faster and more readily available. All the milk coagulants fall under four categories: aspartate, serine, cysteine, and metalloproteases. and each has its specific mode of action (Ben Amira et al., 2017; Shah et al., 2014). However, aspartate protease-type coagulants are used during cheese production that cleaves milk k-casein fraction from C-terminal at the Phe_{105}-Met_{106} bond. Such plant-based coagulants also improve the nutritional status of the cheese produced (Silva & Malcata, 2005). Of many suggested plant coagulants, thistle plants like *Cynara cardunculus* also known as cardoon-derived coagulants were found to be the best-studied example for milk coagulation. The cheese produced using the cardoon-derived coagulant had buttery, creamy, and soft-textured characteristics (Alavi & Momen, 2020; Feijoo-Siota & Villa, 2011; Ordiales et al., 2013). The following Table 13.1 summarized different varieties of cheeses produced using milk coagulant from cardoon.

The plant-derived milk coagulant has certain disadvantages like, due to over proteolytic activity of the enzyme, it imparts bitterness to the cheese. Also, decreases in yield and distorted texture have been associated with the use of plant-derived coagulants. So a more enhanced and improved source for rennet enzymes has shifted towards native rennet-producing enzymes or the use of genetically altered microorganisms (Shah et al., 2014).

The microbial-based milk coagulant can be isolated, or genes for the coagulant can be manipulated, to get a target specific coagulant in order to avoid or overcome the bitterness to the cheese as in the case of plant-derived milk coagulants. The various microbes used for the production of rennet enzymes are summarized in Table 13.2.

13.4.8 Trend of diversified microbes for cheese production

The genetically modified microbes have been utilized to ensure starter culture and nonstarter culture improvement in order to accomplish a given task which would normally be failed by a single strain. Also, genetically modified microbes have resistance power from the attach of bacteriophage. The bacteriophage contaminations are common in dairy industries which kills the microbes of industrial importance through lytic or lysogenic pathways. Many biotechnological tools like mutation, gene transfer through conjugation and transformation have been applied to create microbial strains of economical

TABLE 13.1 Cheese produced by different milk using *Cynara cardunculus* derived milk coagulant.

Cheese produced	Type of milk used	References
Torta del Casar	Ewe	Ordiales et al. (2013)
Tallaga	Buffalo	Abd El-Salam et al. (2017)
Djben	Ewe	Mouzali et al. (2003)
Serra	Ewe	Macedo et al. (1993)
Caciotta	Blend of Cow, Ewe, Goat	Aquilanti et al. (2011)
Camembert-type	Cow	Zikiou and Zidoune (2019)

TABLE 13.2 Different bacterial and fungal strains used for milk coagulant production in cheese production.

Source	Strains	References
Bacteria	*Bacillus subtilis*	Meng et al. (2018)
	Bacillus stearothermophilus	Ahmed et al. (2016)
	Bacillus amyloliquefaciens	Guleria et al. (2016)
Fungi	*Mucor mucedo*	Abou Ayana et al. (2015)
	Rhizomucor miehi	Bailey and Siika-aho (1988)
	Rhizomucor pusillus	Andrén (2011)
	Cryphonectria parasitica	Andrén (2011)

efficiency. Mutations work through the exposure of the microbial strain to mutagenic compounds. But the use of mutation is still seldom due to the fact that it results in disrupting and undesirable characteristics. Next is conjugation, where plasmid or genomic strands carrying desirable characters are transferred to other microbial strains through close contact. The use of conjugation has its own cons like the method is feasible with only closely related species. The final way to modify microbial strains is through the use of transformation. The transformation deals with the pure DNA strains having the capability to be edited through knocking out undesirable genes and the insertion of target gene interest into the desired strains. The transformation is feasible with intra- as well as inter-microbial species, that is, the exchange of interest gene can occur in nonrelated microorganisms. As such, no legal regulations for genetic manipulation in GRAS status microbes are required, as these are beneficial and nonharmful strains used in food industries. The use of genetic engineering in cheese production has been limited to proteolytic enzyme production. Enhanced proteolytic enzyme production through gene manipulation has increased the curdling process as well as sensory characteristics of the cheese during ripening.

Earlier, calf rennet coagulant was used for the proteolysis process. But due to less availability and high cost was soon replaced by plant-based coagulants. However, satisfactory results were obtained using the plant-based coagulants but it over-processes the proteolysis, giving cheese undesirable characteristics. The microbial-derived coagulant served the best example for the biosynthesis of rennet. There was the fungal origin for the rennet enzyme but was less effective than the calf rennet. The genetically engineered strains showed high rennet production with high thermal stability and chymosin content. Such properties had control over the proteolysis process. In practice, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Aspergillus niger*, *Lactobacillus lactis*, and *Escherichia coli* have been successfully genetically modified to produce proteolytic enzymes (Garg & Johri, 1995; Geisen & Holzapfel, 1996). Guldfeldt et al. performed a proteolytic gene transfer of *pepN* gene from plasmid pFG2, *pepV* gene from *Lactococcus lactis* subsp. *lactis*, and *pepO*, *pepC* genes from *L. lactis* subsp. *lactis* CHCC377 to *L. lactis* subsp. *cremoris* MG1363. Enhanced proteolysis that liberated FAAs contributed to texture and sensory characteristics to full-fat cheddar cheese preparation without any significant loss in the yield (Garg & Johri, 1995). Tyagi et al. investigated the production of mozzarella cheese using an inducible expression of buffalo prochymosin in recombinant yeast *Pichia pastoris*. The yeast showed increased production of chymosin rennet which was used to prepare cheese that had a more enhanced sensory and texture characteristics with low moisture content than the cheese produced using meito rennet (Tyagi et al., 2017). The genes for proteolytic activity *pepN*, *pepC*, *pepX*, *pepI* were transferred from *Lactobacillus helveticus* to a food-grade starter culture of *L. lactis* reducing the ripening process period significantly through enhanced proteolysis and improved sensory parameters in cheddar cheese production (Joutsjoki et al., 2002). Another microbial-based rennet isolated from rice wine was *Saccharomyces cerevisiae* which enhanced primary and secondary proteolysis that contributed to the ripening of cheddar cheese. The cheese had a soft texture along with higher volatile compounds and FAAs that contributed to the flavor of the cheese. The bitterness due to higher FAAs was attenuated by other sweet, umami, and tasteless FAAs (Zhao et al., 2019). The domestication of cheese using molds was just a mere theory back in the past in order to give the cheese a novel flavor during ripening. The process is controlled by humans for diversifying cheese for its market potential. The same approach has been utilized in the production of feta, Camembert, and Brie cheese using different nonstarter culture *Penicillium commune Penicillium* spp., *Penicillium biforme*, and *Penicillium fuscoglaucum* for giving an iconic color, flavor, and texture. The molds grow as filaments on the surface of the cheese and gave a fatty aroma due to the proteolysis and lipolysis process which was intriguing as the ancestral strains produced musty and earthy aroma. The fatty aroma cheese was the most-liked cheese to many consumers. Both the molds used have a strong correlation to *Penicillium camemberti* lineage. The mold's domesticated cheese had an additional function of inhibiting other mold pathogens, thus contributing to enhanced shelf life to the cheese (Bodinaku et al., 2019; Ropars et al., 2020).

13.4.9 Trend of fortified cheese

The incorporation of bioactive ingredients like probiotics, prebiotics, vitamins, minerals, and macromolecules in cheese to increase its nutritional value has increased the interest of the manufacturer. Such fortification in cheese is due to the consumers' need to maintain a healthy life and wellbeing. Also, the sole purpose of the fortification is to ensure the safety and retention of bioactivity of the ingredients added during the process or storage of the cheese. Such fortified cheeses are widely accepted and consumed all over the world.

13.4.9.1 Probiotic and prebiotic fortified cheese

Cheese has known for the delivery of probiotics and many recent strategies have been involved in maintaining the minimum dose of probiotics (10^6-10^7 CFU/mL) recommended by the FDA. The viability of probiotics in cheese is

TABLE 13.3 Incorporation of probiotic strains in different kinds of cheese.

Immobilizing carrier	Probiotic strains used	Cheese formed	References
Wheat bran	*Lactobacillus casei*	Feta-type cheese	Terpou et al. (2018)
Lamb rennet paste	*Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium lactis*	Pecorino cheese	Santillo et al. (2012)
Alginate	*Lactobacillus paracasei* spp. *Paracasei*	Mozzarella cheese	Ortakci et al. (2012)
Alginate	*B. longum*	Cheddar cheese	Amine et al. (2014)
Rennet	*L. paracasei*	Feta	Kia et al. (2018)
Alginate	*Lactobacillus rhamnosus*	Cream cheese	Ningtyas et al. (2019)
Fructo-oligosaccharides	*L. acidophilus B. lactis*	Petit Suisse cheese	Cardarelli et al. (2007)

maintained through their enhanced growth rate of 10- to 100-fold upon storage (Ganesan et al., 2014). *Lactobacillus rhamnosus*, *Lactobacillus pentosus*, *Leuconostoc mesenteroids*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Leuconostoc lactis*, and *Lactobacillus paracasei* are some of the strains which are included in QPS and are readily used for the fortification of different kinds of processed cheese (Pino et al., 2019). Widely, there are two methods through which probiotics are kept viable for a longer period of storage: gel entrapment and microencapsulation. Both of the methods deal with probiotic cell immobilization which currently has become an expanding research due to the feasibility for technical and economical purposes. As we know, the probiotics offer health benefits against many gastrointestinal tract diseases, and they also enhance the quality of the cheese in terms of sensory as well as organoleptic parameters. The probiotic-coated films were also used for the delivery of probiotics to the cheese. The following Table 13.3 summarizes the use of different immobilizing carriers for the entrapment of probiotic strains used for the development of varieties of cheese.

According to one such report, two alginate-based edible films were prepared that were coated with probiotic strains of *Lactobacillus acidophilus* and *Lactobacillus helveticus*, respectively. The *L. helveticus*-coated edible alginate film showed significant delivery into the cheese. However, both the edible films maintained their viability until 10 days of storage and had significant inhibitory effects on the coliforms. This was due to the production of lactic acid that decreases the pH and also, probiotic-coated films maintain the moisture content of the cheese that stabilizes the overall texture (Olivo et al., 2020). In another report, the addition of probiotic strain *Streptococcus thermophilus* aided in the cheese production through enhanced lactic acid production and sensorial characteristics. The ability to withstand high temperature, the probiotic strain was added during the process of white cheese production. The cheese produced had a decreased pH range due to lactic acid formation that decreased the risk of contamination, and the probiotic strain did not affect the overall composition of the cheese. The viability of the strain in fresh white cheese was also maintained at a higher rate during 28 days of storage (Yerlikaya & Ozer, 2014). Another report provided the addition of probiotics *L. acidophilus*, *L.s casei*, *L. paracasei*, and *Bifidobacterium animalis* into cheddar cheese preparation, remaining viable after 280 days of storage. The probiotics did not have any negative effect on the growth of starter cultures for the fermentation. The most striking evidence given was that the high-fat content of the milk used for cheddar cheese production was necessary for the survival of probiotics (Ganesan et al., 2014). Yet another report, the production of Squacquerone cheese with starter culture *Streptococcus thermophilus* and a probiotic strain *Lactobacillus crispatus*. The probiotic addition to the cheese had a positive effect on the growth of the starter culture. The overall cheese texture in terms of creaminess was also enhanced and widely acceptable. The cheese with probiotics had an enhanced storage efficiency as it inhibited the growth of yeats, Enterobacteriaceae, and molds, and even positively modulated the gut microbiome of the humans (Patrignani et al., 2019). The probiotic Coalho cheese was produced using goat milk with standard cultures along with probiotic strain *Lactobacillus acidophilus*. The cheese was rich in conjugated linoleic acid which had no effect on the viability of the probiotic strain and remained viable at high numbers during the storage period of 60 days (Dos Santos et al., 2012).

The viability of probiotics in any product is the major challenge faced by many industries. An alternative has been widely exploited—the use of prebiotics for maintaining the viability for a longer time period. Prebiotics are nondigestible components and the widely used prebiotics are inulin (fructans), oligofructose, lactulose, galacto-oligosaccharides, and wheat bran (Cardarelli et al., 2007; Karimi et al., 2015; Terpou et al., 2018). The prebiotic controls the fermentation. The prebiotic fructo-oligosaccharide (FOS) along with honey used to encapsulate probiotic strains of *L. acidophilus* and *B. lactis* in the formation of probiotic Petit Suisse cheese. Apart from exhibiting beneficial aspects to humans, the probiotics strain enhanced the fermentation rate, thus decreased the time period of fermentation (Cardarelli et al., 2007). The prebiotic wheat bran used for immobilization of probiotic strain *Lactobacillus casei* showed a high viability rate even after 6 months of storage. During the ripening of the cheese, wheat bran-immobilized *L. casei* showed inhibition of food-borne pathogens due to low pH by lactic acid production. Also, the wheat bran-immobilized *L. casei* had enhanced the production of volatile compounds through fat degradation in the feta-type cheese (Terpou et al., 2018). Inulin is a prebiotic that has been widely used as a fat replacer in the production of low-fat cheese. The need for low-fat products is a great concern to the manufacturers due to the increased demand for processed food products with dietic and functional properties. However, the low-fat in the product corresponds to poor organoleptic properties, so inulin, being a texturizing agent balances the properties. The inulin of long-chain lengths and high molecular weight forms microcrystals when mixed with water or milk, thus, giving a creaminess texture to the product and also maintaining the organoleptic properties similar to high-fat products. The inclusion of inulin in the processed cheese spread using cheddar cheese was documented. The inulin incorporation was 0%, 4%, 6%, and 8%. The high level of inulin incorporation resulted in decreased water activity and increased moisture content due to the high solid content in the cheese. The high level of inulin also decreased the sensory properties like softness and spreadability. However, there was no significant change in body, texture, flavor, or color of the processed cheese spread (Giri et al., 2017). The addition of inulin as a prebiotic in low-fat UF (ultrafiltrate) soft cheese enhanced the moisture and ash content and also the sensory characteristics, but the organoleptic properties were decreased by the prebiotic addition. However, inulin maintained the viability of the probiotic strain *L. acidophilus* and *Bifidobacterium* spp. in the cheese (El-Baz, 2013). The addition of 2% inulin along with two probiotic strains *Lactobacillus rhamnosus* and *Bifidobacterium lactis* during the production of low-fat Domiati cheese had a positive effect on the proteolysis and enhanced the sensory and texture characteristics. Additionally, inulin maintained a high count of probiotics during the storage period (Abd-Rabou et al., 2016). The FOS positively modulated the growth of probiotic strain *L. paracasei* during the production of symbiotic semihard cheese. The addition of prebiotic and growth of probiotic had no significant physio-chemical change on the cheese characteristics (Langa et al., 2019). Prebiotics such as inulin, lactulose, and FOS has been used for maintaining the viability of the probiotic strains *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus reuteri* during the production of cream cheese. Among the used prebiotics, lactulose and FOS had prolongation property towards probiotics viability and the lactulose offered protection to the probiotics from the changing cheese environment upon ripening. Lastly, the cheese produced did not have any negative or detrimental sensory and organoleptic off characteristics (Speranza et al., 2018).

13.4.9.2 Vitamin-fortified cheese

The vitamins are organic molecules acting as a micronutrient for the proper functioning of human metabolism. To date, vitamin D has intrigued the cheese production research because of varied functions. Vitamin D is a fat-soluble vitamin. The two major forms, D_2 (ergocalciferol) and D_3 (cholecalciferol), are taken through diet in an inactive form which becomes active in the liver or kidney. The human form of vitamin D is D_3 which upon sun exposure changes to an active form. This active vitamin D is 1,25-dihydroxyvitamin D that favor calcium as well as phosphorus absorption which aid in bone mineralization and also treat certain diseases like cancer, diabetes, osteoporosis, and autoimmune disorders. The fortification of vitamin D in cheese needs to subdue the requirement of vitamin to the body. One cup of milk provides 100 IU vitamin D, which is quite low in meeting the daily requirement of 600 IU. Also, vitamin D fortification does not affect the quality of the cheese produced. The vitamin D fortified cheddar cheese, using an emulsion, did not affect the sensorial effect and texture of the cheese, and also had an elevated shelf life of around 9 months. Two hundred or 400 IU of vitamin D_3/cheese serve was maintained at the end of the storage period (Ganesan et al., 2011). The incorporation of vitamin D in milk of cheddar cheese has successfully experimented using oil in water emulsion of sodium caseinate, calcium caseinate, and nonfat dry milk. The cheddar cheese of high vitamin D was maintained to provide 280 IU of vitamin/per cheese serving (Tippetts et al., 2012). The incorporation of vitamin D did not affect the composition nor the sensory properties of the cheese. Vitamin D was added to cottage cheese production after the drainage of the whey to avoid the washout of the vitamin. An emulsifier cream (145 IU vitamin/g of cream) was used for mixing the vitamin into the cheese and the final cheese product had 51 IU vitamin D/g of cheese (Crevier et al., 2017). The fortification of vitamin D in cheese did not have any off-flavors nor loss of vitamin during production and storage (Upreti et al., 2002).

13.4.9.3 Mineral-fortified cheese

Among all the minerals, zinc is readily available in food sources like meat. For humans, zinc is a necessary mineral for most of the catalytic enzyme activity. Also, it has a high demand for beneficial immune function, protein, and DNA synthesis, wound healing, and cell division processes. The recommended dietary allowance (RDA) for zinc in men and women is 11 and 8 mg, respectively. The intake is increased during pregnancies, childhood, and adolescence. Collectively, zinc is required for normal health wellbeing and its maintenance. However, people with vegan and vegetarian lifestyles do not meet the RDA for zinc, so it becomes a necessary task to ensure the adequate intake of zinc through different sources to eradicate deficiencies. Thus, fortified cheese with zinc offers more benefits for providing adequate intake of zinc. There are different zinc salts that have GRAS status, which includes zinc sulfate, zinc stearate, zinc chloride, zinc gluconate, and zinc oxide. The incorporation of zinc during cheese production does not interfere with the growth of starter culture. In the research of Kahraman and Ustunol, zinc fortified cheddar cheese had no negative effect on the starter culture. However, intriguingly, the cheese had higher ash, fat, protein content, and low moisture content as compared to the control cheese that gave hardness to the cheese (Kahraman & Ustunol, 2012).

Another mineral, iron, is also important for human growth and maintenance. The deficiency of iron leads to anemic conditions where the body cannot generate enough red blood corpuscles. The prevalence of anemia has increased at a higher rate (Howson et al., 1998), so the need for processed food with mineral incorporation is of great concern. The fortification of cheese with iron enhances the nutritional value. Iron-fortified cheddar cheese in the form of ferric chloride, ferric-casein, ferric-WP from skim milk enhanced the dietary intake of the iron, and was a revolutionizing step for demolishing its deficiencies. Also, the iron-fortified cheese prevented any oxidized off-flavors, and maintained fat and protein content as compared to unfortified cheese (Zhang & Mahoney, 1991).

13.4.9.4 Spices and herb-fortified cheese

Spices, herbs, and other condiments are regarded as flavoring agents and are widely used in many food products. Fortification of spices and herbs into cheese further diversifies the quality, texture, sensorial, and organoleptic parameters. Table 13.4 summaries the common spices and herbs used for the fortification of cheese. These additives are added after the whey drainage and are thoroughly mixed. The additives also improve the microbial quality of the cheese.

The ginger-fortified cheese enhances the growth of starter culture *L. lactis* spp. *lactis* and *L. lactis* spp. *cremoris* strains along with improved flavor and texture of the cheese. The fortified cheese, produced using herbs, has an extended shelf life and enhanced flavor and texture (El-Aziz et al., 2012). Garlic is a potent inhibitor of food spoiling pathogens, and fortification of cheese using garlic showed improved inhibition of such pathogens. The garlic used at 2% showed productive growth of starter and nonstarter cultures and also improved the texture and sensorial properties of the cheese (Farrag et al., 2019). The combination of spices and herbs like thyme, mint, cumin, basil, garnet, estragon, fennel, and bay were used for the fortification of mozzarella cheese. These additives had a slightly negative effect on the growth of starter culture *Streptococcus salivarus* spp. *thermophilus*, *Lactobacillus delbreuckii* spp. *bulgaricus*, and *Lactobacillus helveticus* during the last days of the storage period. However, the cheese that was produced had an improved textural, sensorial, ripening index (Akarca et al., 2016).

13.4.9.5 Essential oil-fortified cheese

Due to increasing cheese spoilage, various approaches have been used to avoid microbial spoilage like improved pasteurization, use of nitrates, lysozyme, etc., but none of them seems to be an ideal approach, as it may increase the production cost. Essential oils have proven to be an alternative in terms of their wide range of microbicidal effects that can be used for the fortification of cheese in a way to dismantle the spoilage and enhance the shelf life of the product. Additionally, the use of essential oils also has advantages such as it gives more flavors and aromas to the cheese which would attract the consumers with more varieties. The essential oils also do not interfere with the growth of starter

TABLE 13.4 Common spices and herbs used in cheese fortification.

Spices	Herbs
Paprika Habanero Chipotle Jalapeno Cloves Cumin Onion Garlic Ginger	Parsley Basil Nutmeg Oregano Thyme Sage Rosemary

TABLE 13.5 Different essential oils used in fortification of cheese.

Cheese	Essential oil source	Inhibition pathogens	References
Pressed ewe's	*Melissa officinalis*, *Ocimum basilicum*, *Thymus vulgaris*	*Escherichia coli*, *Clostridium tyrobutyricum*, *Penicillin verrucosum*	Licon et al. (2020)
Mozzarella	Thyme and rosemary essential oil with sodium diacetate	*Listeria monocytogenes*	Han et al. (2015)
Argentinean	Oregano essential oil	Aerobic mesophilic, Enterobacteri, molds, and yeasts	Marcial et al. (2016)
Coalho	Thyme essential oil from *T. vulgaris* L.	*Staphylococcus aureus*, *L. monocytogenes*	De Carvalho et al. (2015)
Cow milk	*Satureja hortensis* essential oil	*S. aureus*	Alexa et al. (2018)

culture and nonstarter culture. There are many kinds of cheese that have been fortified using essential oils that are summarized in Table 13.5.

13.5 Conclusion and future aspects

Cheese has diverse microflora which contributes to a wide variety of cheese. With intensive interaction of starter culture, milk microbiota and secondary culture fail to provide economically good quality textured cheese. The strain selection does not provide control of cheese production over the industrialist. New strain isolation for cheese production is not economically feasible with small industries. However, new approaches like genetic engineering and manipulation of genes in existing strains offer more advantages in terms of cheese yield to achieve desirable properties. Also, the key principles during cheese production such as maintaining consistent milk composition with a low mesophilic count, monitoring of the process to avoid undesirable attributes, and storage facilities have never changed, which makes the cheese plant successful.

The future need for improving the cheese industries need to focus more on using tools like genomics and proteomics for a better understanding of biochemical and microbiological aspects of the cheese. Such tools will further improve the knowledge for different approaches of fortification of cheese for enhanced texture and functionality. Further research needs to be focused on cheese production using molds as starter culture, as very little is known. Also, more knowledge regarding the domestication of cheese using another mold needs to be researched, for creating further varieties of cheese with great economic potential to industries.

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Chapter 14

A new generation of sustainable life forms of milk kefir grains produced from freeze-dried microbial isolates: observational study of grain behavior in an experimental system

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14.1 Introduction

Milk kefir grains (KGs) consist of discrete clusters of bacteria and yeasts existing in symbiotic association, and encapsulated within a matrix of extracellular polymeric substance (EPS) of bacterial origin (Douglas, 1911; Hui & Özgül Evranuz, 2012; Rattray & O'Connell, 2011). The potential health benefits from consuming KGs regularly are the subjects of numerous research. In particular, their regulatory functions on the immune system and other tissue organs has been published (Farnworth & Mainville, 2008; Leite et al., 2013; Simova et al., 2002). There are various methods available for the preparation of milk kefir throughout the world. A milk kefir culture can be started using microbial isolates present in a freeze-dried starter and/or lyophilized grains, but this type of culture can only be used for the production of three or four batches of fermented milk resulting in a flavor profile different from when fresh grains are used as a starter for the milk culture.

Commercially available kefir can be found in pasteurized liquid forms in many stores; it contains a few select strains of bacteria and yeasts, whereas traditional kefir produced at home, contain multiple strains (>60) and many other compounds occurring during fermentation. A detailed description of the strains most commonly found in real kefir has been published (Bottazzi & Bianchi, 1980; Chen et al., 2009; Garrett et al., 2008; Gulitz et al., 2011; Lu et al., 2014; Marshall et al., 1984). Bacterial species including Lactobacilli, but also Lactococci, Streptococci, *Leuconostoc*, and also, yeast species mainly of the genera *Kluyveromyces*, *Candida*, and *Saccharomyces* predominate. Morphologically, the grains are characterized by irregular clusters of bacteria and yeasts, and sometimes molds, forming symbiotic communities (Garofalo et al., 2015; Maeda et al., 2004; Özer, 2014; Vu et al., 2009). The grains are white to off-white and when placed in milk, they swell. The most active grains generally are found near the surface, and store nutritious fluid in their hollow interior. They tend to clump together and expand. Over time, the formed CO_2 is released from the grains in discrete bursts.

Photographs of grains collected from all other the world have been published (Farnworth, 2005; Habibi et al., 2011; Kritee, 2007; Mistry, 2004; Yaman et al., 2006). The grains can propagate indefinitely as long as the culture is fed with whole milk on a regular basis. The yeast component is essential, although its proportion is much lower than that of bacteria ($\sim$30% and 80%, from reference Magalhães et al., 2011). The nutritional value of kefir is based on the use of milk as a main ingredient and the combined effects of primary and secondary fermentation. Apart from the mode of handling and many other variables, the quantity of grains and their fermentation potential determine the quality of the kefir product obtained. Primary fermentation occurs at room temperature (18°C−23°C) during which time there is

[†] I'm honoured to have known Ms. Brigitte Richard who is unfortunately not with us anymore. God rest her soul in peace!

-Joginder Singh

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consumption of lactose and production of heat energy, water, CO_2, organic acids and ethanol along with a white gelatinous substance from the EPS-producing strains of bacteria.

Texture and flavor develop during the secondary fermentation when the kefir is refrigerated for 2−3 days. During this time, aromatic substances develop through microbial enzymatic activity. Some metabolic products are volatile while others are processed further and assimilated (Douglas, 1911; Farnworth & Mainville, 2008; Hui & Özgül Evranuz, 2012; Rattray & O'Connell, 2011; Simova et al., 2002).

Commercial starters based on microbial isolates have limited usage and cannot normally produce authentic kefir grains (Fuqua et al., 1996). To make authentic kefir, one needs a starter containing KGs (Bergman, 2001; Katz & Pollan, 2012; Leite et al., 2013; Magalhães et al., 2011; Seher, 2014; Sieuwerts et al., 2008). Given that no one has yet succeeded in reconstructing KGs from microbial isolates (Farnworth & Mainville, 2008; Oberman & Libudzisz, 1998; Stadie, 2013; Yaman, 2006), the possibility of using component strains as starting material was explored with the aim of producing authentic KGs and in addition, examine their temporal transformation and natural behavior as they grow and propagate.

The project started in the summer of year 2012. Specific aims of this research were (1) to provide direct experimental evidence of formation of KGs from microbial isolates using a milk pouch system; (2) allow the smaller grains to propagate indefinitely in milk; and (3) culture the larger grain specimens in a hybrid experimental culture model (ECM) system to study their natural behavior. Over the years, it became evident that the grains have gained resilience allowing them to be easy to propagate. Ultimately, this work aimed at contributing to the existing knowledge on KGs as part of a complex and diverse microbial ecosystem.

14.2 Material and methods

14.2.1 Supplies

Containers used in this study were made of food-grade material. Clear glass jars (dimension: 9.5 × 7.0 cm; capacity: 300 mL) were purchased from Euro Cuisine Inc. (Commerce, CA, USA). Frigoverre glass pitcher (dimension: 15.0 × 7.0 cm; capacity: 750 mL) was purchased from Bormioli Rocco Glass Co (Fidenza, Italy). Food storage container with lid, 9.5 L-capacity from Rubbermaid Inc. (Hunterville, NC, USA) served as a "microclimate" chamber. Precut ultrafine cotton cheesecloth measuring 2.0 × 3.0 cm, and butcher's twine were from Regency Wraps, Inc. (Dallas, TX, USA). A set of nylon mesh strainers (mesh size 0.8 mm) was from Harold Import Co Inc. (Lakewood, NY, USA).

14.2.1.1 Ingredients

Grade A pasteurized homogenized cow's milk with vitamin D, and instant nonfat milk with vitamins A and D were purchased from Jerseymaid Milk Products, Inc. (Vernon, CA, USA) and Carnation Nestle SA. (Vevey, Switzerland), respectively. Organic brown sugars of various sources were purchased at a local health-food store, and consisted of evaporated sugar cane juice, coconut palm sugar, blackstrap molasses (a viscous by-product of sugar cane and a good source of iron, potassium, calcium and magnesium), and candy sugar crystals derived from beets. Organic coconut water, Baker's yeast, Japanese green tea (parts used such as whole leaves, root, stem, and bark), dried fruits (figs, dates, raisins, cranberries) and fresh fruits (bananas) were from local stores. The freeze-dried kefir starter containing microbial isolates in powder form was purchased from Lyo-San Inc. (La Chute, Quebec, Canada). Each packet of 5 g contains skim milk powder and ascorbic acid for cryopreservation, as well as at least 3 billion lactic bacteria and 50,000 yeasts (cell count per gram). According to the manufacturer's product information, the major select strains in the kefir prepared from this starter, are *Streptococcus lactis, Streptococcus diacetylactis, Streptococcus cremoris, Lactobacillus casei*, and *Lactobacillus acidophilu*s, along with yeast strains such as *Saccharomyces lactis* and *Saccharomyces cerevisiae*. In addition, there may be other bacterial species derived from the original grains used to prepare the starter (Chen et al., 2009; Garofalo et al., 2015; Garrett et al., 2008; Gulitz et al., 2011; Habibi et al., 2011; Kritee, 2007; Lu et al., 2014; Maeda et al., 2004; Mistry, 2004; Özer, 2014; Vu et al., 2009). The brewer's yeast suspension from White Labs, Inc. (San Diego, CA, USA) (http://www.whitelabs.com/yeast/wlp001-california-ale-yeast) contains *S. cerevisiae*, California Ale yeast, and WLP001 ale yeast used to supplement the culture and stimulate growth (Bhat & Bhat, 2011; Garrote et al., 2010; Guzel-Seydim et al., 2011; Hayek et al., 2013; Iwasawaa et al., 1982; Motaghi et al., 1997; Ninane et al., 2005; Păucean & Socaciu, 2008; Păucean, Rotar, Jimborean, Mudura, & Socaciu, 2009; Pop et al., 2014; Zajšek & Goršek, 2011).

14.2.2 Methods

14.2.2.1 General

This is not a classical experiment in a laboratory setting. The cultures were observed at room temperature (in the range of 28°C−30°C) and relative humidity (in the range of 70%−75%) in a private kitchen area, under semicontrolled conditions with full precautions taken to let natural air currents circulate and minimal handling in an effort to reduce the risk of contamination (Leite, 2013).

14.2.2.2 Reconstruction experiments

The experiment consisted of two phases. In the preliminary phase, milk kefir was prepared according to manufacturer's instructions (http://www.yogourmet.com/usa/kefir-starter-culture.php). Briefly, a packet of 5 g of freeze-dried powder makes 1 liter of kefir. Pasteurized milk is heated up to 82°C and cooled down to room temperature. The starter powder is stirred into the milk and left to incubate for 24 hours at room temperature (in the range of 28°C−30°C). Kefir inoculum from the previous batch (up to 10%, v/v) is added to start a new batch. This starter has limited usage because it does not contain KGs; only three or four batches can be made out of a packet of 5 g, which prompted us to continue the quest for KGs production.

Subsequently, 10 g of freeze-dried powder (two packets) and 15 g of candy sugar crystals, as a source of fermentative sugars, were placed into a pouch, securely tied up in place, and then, partially immersed in pasteurized milk (1 L), previously heat-treated as noted earlier. The same pouch was transferred to fresh milk, once daily over 72 hours. At each transfer, reserved fermented milk from earlier was added (10%, v/v) to boost the next fermentation cycle. The experiment was carried out in triplicate.

After the last 24-hour incubation period, the granular formations present in each pouch were collected in a fine-mesh strainer, and briefly (<5 minutes) preserved in fermented whey from earlier. The smaller granules, typically less than 1 mm were immediately tested for their ability to propagate in milk whilst the larger grain particulates were tracked separately in an ECM model described in the following sections.

14.2.2.3 Preparation of kefir based on traditional method

The production of authentic kefir is very simple, and consists of a two-step fermentation process (Leite et al., 2013). A simplified method for routine maintenance is used. Briefly, the thickest part of kefir (inoculum) is scooped out (about 2 tsp) and place it in a clear glass container; then cold milk is added using an approximate inoculum-to-milk ratio of 1/5 ratio, making sure to raise the milk container at a certain height while pouring to make the milk frothy. Stir (optional). Let incubate 1 or 2 days at room temperature until some whey is visible at the bottom and repeat the process. Keep leftovers in the fridge for the second fermentation step. The kefir is ready after a couple of days or more.

For long-term preservation, spare grains were dehydrated by rolling them over on a clean paper towel until the paper looks dry, and then mixed with an excess of dry milk powder by tossing into a plastic bag. By using this procedure, the grains are viable when stored for up to 18 months at −20°C.

14.2.2.4 Experimental model

To detect morphological features and collective motions otherwise not visible in milk, the larger grain specimens ($n = 12$) were used as initial inoculum in a new ECM culturing system. The culturing process is described next.

A whey-molasses solution was made of 500 mL of 1% molasses solution and prepared fresh on each day by combining 5 mL of molasses with 450 mL of filtered water and fermented whey from the milk culture (1:1, v/v). This solution served as fermentation medium with 15% sugar content (w/v). A 100-μL suspension ($\sim 1 \times 10^9$ cells) of Brewer's yeast was used during the third week to boost yeast content and potentially stimulate growth of KG cultures.

The addition of fresh milk grains twice weekly and fruits every other week was for culture enrichment. The grains were repeatedly transferred, and given fresh fermentation medium at 24-hour intervals for 4 months. Between each cycle, the grains were photographed and visually inspected.

Each time, a portion of the fermented medium (10%, v/v) was left to boost the next cycle and the unused fermented medium placed in the fridge for 2−3 days. Sensory measures were valuable to get an estimation of the metabolic demand of the grains in culture. This is important since an imbalance can cause the viability of the KGs to decrease quickly. A medium significantly depleted of nutrients has a distinct yellowish color and cloudy appearance due to high cell density that once observed, is easily identifiable. Sweetness is one of major sensory determinants, and one will notice a change in sweetness over time. The sense of smell is valuable to identify certain types of volatile organic

compounds (VOCs). A strong yeasty smell was a sign that yeasts had overgrown the fermentation broth. Another category of sensory inspection is auditory inspections. When the grains are very active, one notices a fizzing sound. To sum up, the culturing method is relatively simple, but challenging, and utilizing the senses was valuable when monitoring the culturing process.

14.2.2.5 Data capture

A Sony digital camera, Model DSC-W350, was used for image acquisition. Photographing and filming up to 30 frames per seconds was performed during brief periods (<10 minutes) to record grain activity at random moments throughout the day. Images and/or video records were obtained by holding the camera very close to the surface and looking down on the culture, or from the sides through the clear glass while moving the camera around in search of a better view. In addition, short video surveys were taken to capture panoramic views. With this method, it was possible to track multiple grains simultaneously and extract relevant image frames. Background elements in the surroundings were valuable to give a sense of scale.

Daily changes to the grain network were documented. All records were totally dependent on the grains being observable. Grains can be viewed from looking over the culture or through the clear glass without disturbing them. It should be noted that grains don't necessarily always float or face the camera. Because the grains are moving around and the medium is turbid, the visibility will change dramatically throughout the day and one can be prevented from seeing them. The precise time a grain disappears from view can be within a few seconds or minutes, giving a short window of time for observations to be made.

14.2.2.6 Data analyses and reporting

Photographs and video records were analyzed retrospectively. Due to its dynamic nature, the ECM system generated large and unprecedented volumes of data. Such data typically are difficult to analyze and comprehend. All images were retrospectively reviewed in a blinded fashion and carefully examined under magnification (up to 30×). This was a laborious process entirely done manually. Only the most illustrative examples are presented in this manuscript.

14.2.2.7 Morphological features

Biomass description included, but was not limited to, branching network, crack openings, aspects of margins (smooth, irregular), pigmentation (color) and any other patterns of growth (which presumably reflect dynamic microbial population changes in nature). The relative pattern of shading and texture was examined to determine whether different parts of a grain surface are made of new biomass.

14.2.2.8 Collective motions and other behavior patterns

The grains were analyzed in their spatial and temporal context to identify behavior patterns in pairs and/or larger formations, looking at small changes in their position relative to the nearest neighbors that may reflect the possibility of interactions and/or close bonds. The grains are interacting with their surroundings, periodically "diving" to explore deeper layers. It is impossible to predict the location of a grain at any given time.

The position of a select grain was examined relative to neighboring and/or more distant grains, and then checked for periodic shifts in position. The correct performance of this task requires shape discrimination and recognition of visual forms based on the relative development sequences established in the manner described in the above section.

14.3 Results and discussion

14.3.1 Reconstruction results

Microbial isolates in powder form were tested for their ability to regenerate KGs. This was part of an ambitious effort to produce a whole new generation of KGs through the use of a commercial starter which normally cannot be used to produce KGs. It was thought that the active starter cultures sequestered in a milk pouch would have a better chance of forming KGs. Indeed after the last 24-hour incubation period, it was noted that the starter strains had undergone a dramatic transformation into pebble-like structures in their simplest forms, and others with coarse rounded and/or angular segments, varying anywhere from rice- to walnut-size (Fig. 14.1).

Under favorable conditions, nascent grains were able to form spontaneously. Most granules had an almost round or ovoid appearance, typically less than 1 mm wide. Others were noticeably larger (up to about 2 cm). The diverse and

FIGURE 14.1 Results of reconstruction experiment showing the dramatic transformation of starter powder cultures into a whole new generation of kefir grains. (A) Shown from left to right are the experimental set up with milk pouch containing starter cultures (*arrow*) and the newly discovered grains collected in a fine-mesh strainer and suspended in fermented whey. (B) Shown from left to right are a plain view of the distinctively larger grains ($n = 12$) in whey-molasses solution alone and then nestled among fresh supplemental grains (*arrows*). The aged grains measure between 0.5 and 2 cm. The last image shows mixed grains collected into a pile and further examined prior to the next cycle; notice the diverse and complex assemblages; all grains have turned deeper brown due to absorption of molasses. Time-series images at intervals ranging from days to weeks.

complex assemblages were visible as tan-colored masses, likely due to absorption of brown sugar. Similar results were obtained in triplicate.

Those grain particulates showed no visible signs of fermentation activity until later when placed in a whey-molasses solution, at which time they exhibited clear signs of metabolic activity. Activation was inferred from the presence of CO_2 bubbles and extremely slow motions, barely perceptible with the naked eye showing that the KGs are living entities.

The scientific evidence for reconstruction of KGs is finally there. To our knowledge, the present data are the first to show that it is possible to reconstruct KGs out of microbial isolates. This finding provides further support to the studies that have hypothesized microbial surface auto-aggregation as an origin of these structures (Chen et al., 2009; Gulitz et al., 2011).

A combination of events, presumably involving small clusters of bacteria and yeasts, and induction of capsular kefiran by EPS-producing bacterial strains caused this natural transformation phenomenon to occur in just 3 days. It is likely that natural airborne bacteria and yeasts took over at some point to produce symbiotic communities closely interacting together, and able to survive and reproduce. The discovery became the starting point for further studies on KGs as described in the following sections.

14.3.2 Progression of milk culture

The milk culture began by using the smaller grains (newly discovered) as initial inoculum. A typical batch of milk kefir is shown in Fig. 14.2.

The culturing process has generated an abundance of grains resembling cheese curds and/or cauliflower florets (Fig. 14.3). During the course of fermentation, the grains were found to be productive, transforming milk into a beverage presumed to be authentic kefir in accordance with the description of fully-fermented kefir given by Simova et al. (2002).

The results demonstrate the feasibility of microbial isolates to create a new starter. This milk kefir culture has been thriving for over 8 years in the same household.

No attempt has been made to analyze the microbial composition by cultivating the strains under laboratory conditions. A detailed description of the typical strains most commonly found in traditional kefir and aspects of the grains themselves is given in references (Bottazzi & Bianchi, 1980; Chen et al., 2009; Garofalo et al., 2015; Garrett et al., 2008; Gulitz et al., 2011; Lu et al., 2014; Marshall et al., 1984; Özer, 2014).

FIGURE 14.2 Typical batch of milk kefir. After a first fermentation cycle, three distinctive layers are visible. "1" = fermented whey liquid; "2" = smooth gelatinous layer with strands of kefiran-like substance; "3" = large number of KGs embedded in thick curds. Close up views show the expanding network of grains conglomerating on the surface as well as in whey pockets visible through the clear glass. The CO_2 bubbles burst over time forming "eyes." Notice the complex geometry and porous structures with lacy patterns. Some grains resemble cheese curds and/or cauliflower florets. *KGs*, kefir grains.

FIGURE 14.3 Morphological appearance of newly-emerged grains (see recovery procedure). (A) The culture is showing signs of recovery: a very thick, foamy layer has risen to the surface with KGs half-hidden inside. (B) The newly-emerged grains are golden brown and have interesting looking geometric shapes. (C) After passage in milk, three distinctive layers are visible similar to Fig. 14.2. (D, E) The grains have retrieved their typical appearance, showing closely packed clusters of new biomass. No two grains are the same. Note small clusters of new grains (about 5 mm) branching off the main bodies, not yet liberated from the larger grains. Largest grain weighing ~2 g. Total weight of 11 grains ~8 g. *KGs*, kefir grains.

Results from recovery experiments provided additional experimental evidence that grains can spontaneously reconstruct themselves under certain circumstances, and regain inherent ability to propagate. Macroscopic appearance of the newly emerging grains is depicted in Fig. 14.3.

Their productivity suggests a reestablishment of the balance of yeasts versus bacteria and is in line with published observations indicating that supplementation with different minerals and vitamins may promote the growth of KGs (Pop et al., 2014).

14.3.3 Experimental culture model system

An ECM system was developed to study the larger grains ($n = 12$, newly discovered). The grains were cohesive enough to be recovered and studied through repetitive fermentation cycles. The key findings are summarized next.

14.3.3.1 Dynamic transformation

During the first weeks, most of the aged grains grew relatively slow, but then suddenly began developing on subsequent weeks, with increasing complexity, once fresh grains had been introduced into the medium. The image collection database captures the temporal link that connect one form to another. Grains were visually identifiable during photographic

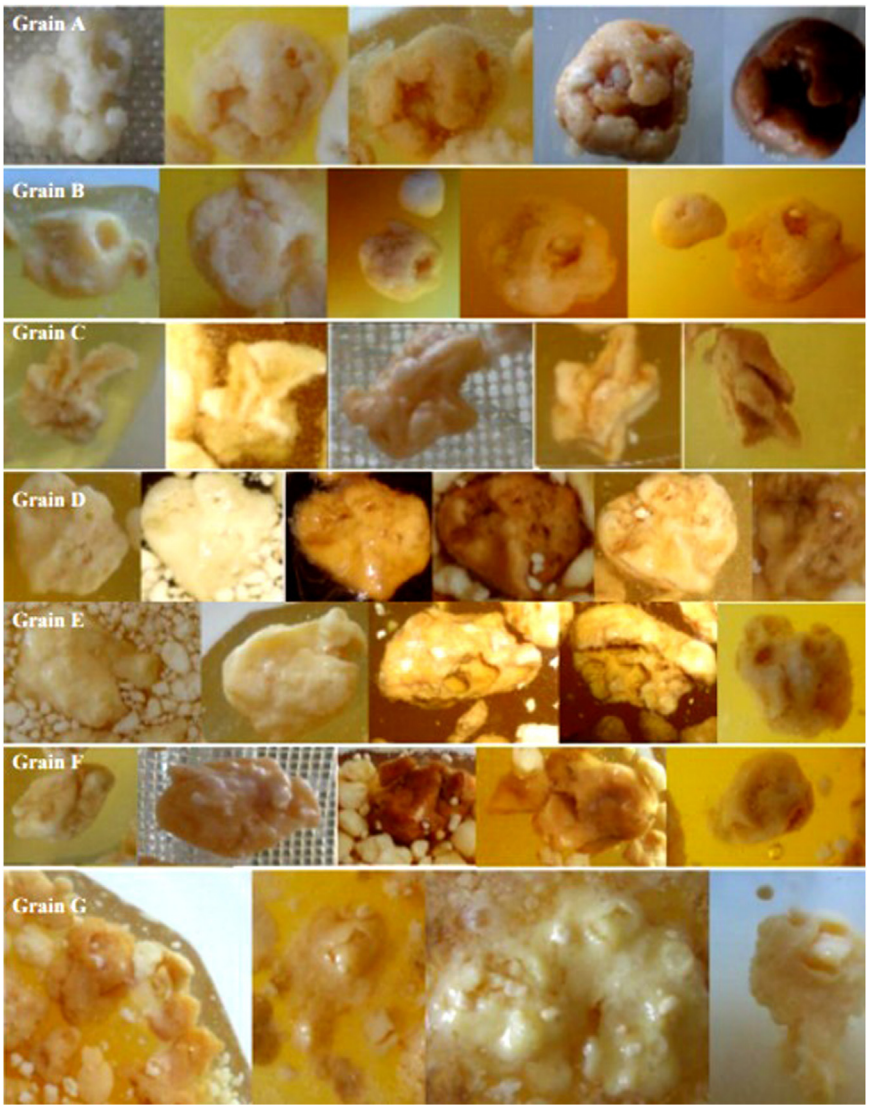

FIGURE 14.4 Reference material from image collection, showing the relative development sequence of select grains established during successive fermentation cycles for the purpose of authentication. Grains labeled A and B (fresh grains) and C through G (aged grains) to facilitate tracking. Most grains have gone through many changes since the time they were first observed. When held in the ECM system, some grains have developed more rapidly on subsequent weeks with increasingly complex forms. Grain G has become larger due to coalescence of neighboring grains. Starting grains measuring ~1.5 cm. Time-series images at intervals ranging from days to weeks. *ECM*, experimental culture medium.

inspection. The grains have different morphological features allowing them to be differentiated from one another (Figs. 14.4 and 14.5).

Collective motion

Their motions are diverse and difficult to predict. They are sensitive to the slightest turbulence created by CO_2 bubbles. Occasionally, the grains initiate some movements that are captivating to watch as exemplified in Fig. 14.6.

They are sensitive to the slightest turbulence created by CO_2 bubbles. Occasionally, the grains initiate some movements that are captivating to watch as exemplified in Fig. 14.6. Some grains are more mobile than others, and seem to regularly patrol their complex surroundings in search of nutrients. Close observations show that grains can shift in different directions, presumably depending on a number of invisible stimuli. They often float at the water's surface gathered in groups or reach deeper layers before resurfacing, presumably seeking new synergistic relationships. Groups of grains move slowly near the surface throughout the day; their extremely slow motions are barely perceptible with the naked eye, but it is clear that they do not limit the area of searching to the surface. Occasionally, they are seen speeding up their movements, twisting or turning over by themselves before diving to reach deeper layers and then come back up. During searching activities, they seem to have precise knowledge about the spatial location of resources and attempt to cover the maximum area possible. This local searching behavior presumably reflects the complex dynamic microbial population changes in nature, influenced by temporal changes in temperature, nutrient gradients and viscosity due to EPS-like substance production—known to be stimulated by the addition of *S. cerevisiae* (Cheirsilp et al., 2003).

Self-propagation

Kefir grains are known to propagate by fragmentation in milk. The clusters liberated from irregular grain surface turn into grains (Kritee, 2007). Finding visual examples of self-propagation in the ECM system was far from trivial in view of the complexity of the culture; remember that a culture may contain hundreds of different grain particulates at any given time. Figs. 14.7–14.9 summarize some of the key events that have occurred during the life cycle of two KGs from their early days through maturation period.

Various visual forms are rendered through a combination of shearing, thinning, and surface covering by new biomass visible on the exterior surface. With each advance of cracks and splits, the fragmented structures can grow back with new biomass filling the gaps. Altogether the data provide visual confirmatory evidence that the grains undergo fragmentation as part of their natural cycle; they are unstable but they are resilient and continue to exist. After partial breakdown, a grain will often sink, at which point further degradation and/or rebuilding of fractured surfaces may begin. As time passes, clusters of biomass are continually released through cracks and splits, leaving remnant structures that are being revitalized to be carried on to the next generations.

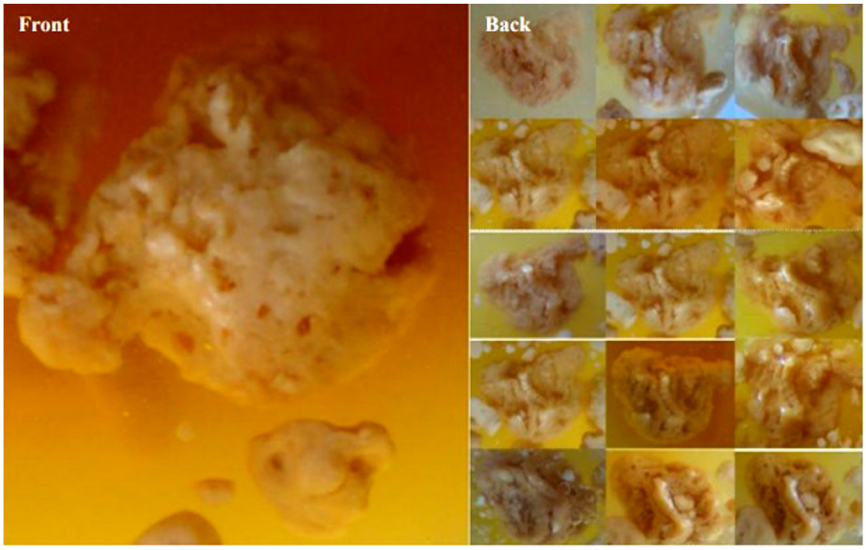

FIGURE 14.5 Complex visual form of a fresh grain in whey-molasses solution (grain labeled H from Fig. 14.4). Close examination shows intricate interlaced surface patterns on either side. The grain floats so one side is exposed ("*front*") and the other side is submerged ("*back*") but visible after the grain has turned over on its own. Smaller grain in the vicinity measuring ~0.7 cm (see Fig. 14.9) gives some sense of scale. Time-series images over a few days.

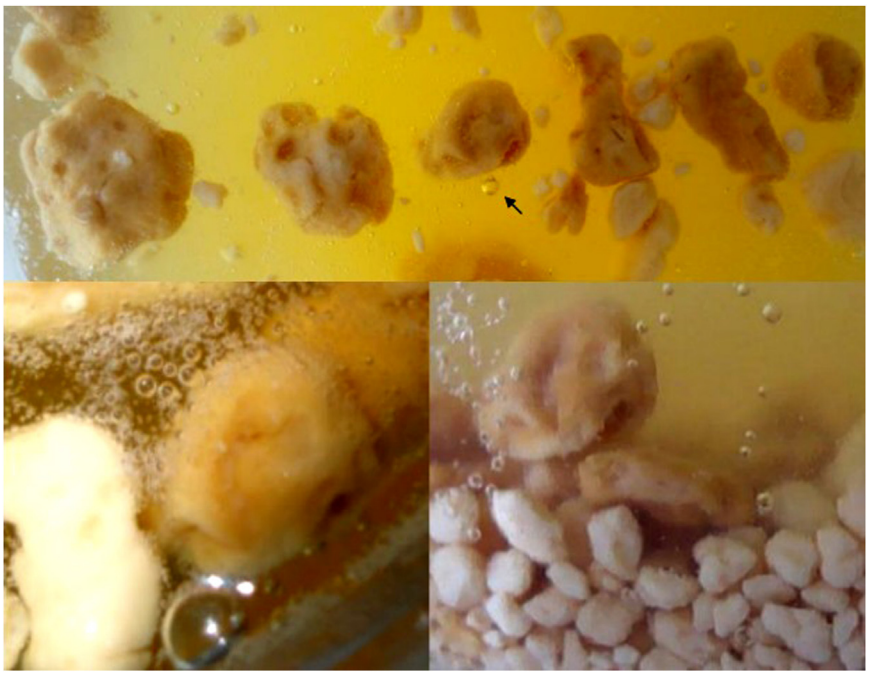

FIGURE 14.6 Illustration of grain mobility (grain labeled F from Fig. 14.4). In the first image, the CO_2 gas is enclosed within the grains so that they float. The most active grain (*marked by arrow*) is seen perusing its surroundings, reaching deeper layers (*3rd image*) and resurfacing (one can notice the stream of CO_2 bubbles released from the inner structures; data not shown). Discharge of the excess CO_2 gas presumably helps the grain regain buoyancy. Also, there is likely enough CO_2 being produced and trapped inside to lift it to the surface naturally. With each motion, the grain tend to tilt in a different direction; the changing orientation and repositioning causes it to display different visual forms that may not be easily identifiable.

FIGURE 14.7 Illustrative example of self-regeneration/propagation into a new visual form (grain labeled E from Fig. 14.4). After apparent weakening and partial breakdown of its structure likely due to turbulence or aging, the grain has moved to deeper layers in the culture. There is apparently enough CO_2 being produced by the new biomass to lift the grain to the surface naturally. Fractured surfaces of isolated grains show evidence of repair/revitalization. Time-series images over 3 weeks. Starting grain measuring $\sim$2 cm. The same phenomenon was observable on the opposite side; see Fig. 14.8 for confirmatory evidence.

14.3.3.2 Behavior patterns

In contrast with the traditional method of growing grains in milk, the ECM system provided colorful scenery with a diversity of moving forms that grabbed the viewer's attention. By looking attentively, it became possible to characterize the culture in terms of how the grains relate to one another as a close community. It is apparent that the grains live in harmony in an ever-changing environment around them. The aged grains appear to continually explore the landscape around them and engage in some form of communication with neighboring grains. Fresh dairy grains do not differ much in their behavior from the aged grains. They also seem to engage with neighboring grains (Fig. 14.10).

The data suggest that grains can detect the proximity of other grains and derive sufficient information to orient in a directional manner. They seem to have precise information about the proximity of other grains and spatial location of resources. This local searching behavior gives them an opportunity to interact with many others in the larger community as they attempt to cover the maximum possible area. This presumably reflects a very transformative world, subject to resources demand due to the diversity of microbial life forms involved in the community.

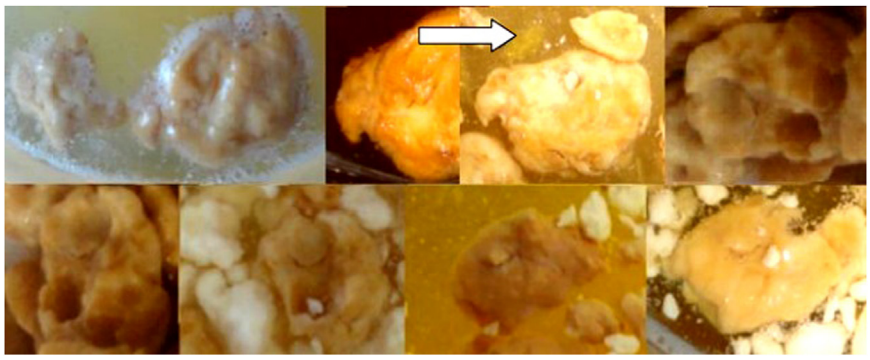

FIGURE 14.8 Evidence of sequential crack growth and splitting events (*arrow*) followed by self-regeneration/propagation into a new visual form (grain labeled E from Fig. 14.4 and Fig. 14.7). Similar clues of repair/revitalization found when the grain has turned over, thereby exposing the other side. It is likely that addition of fresh grains have a stimulatory effect on the culture due to large biomass made available and their numbers allow close contact to take place with aged grains. Time-series images over 3 weeks – the photographs in fourth and fifth positions are identical, except for an orientation difference. Notice the similar contours (compare with Fig. 14.7).

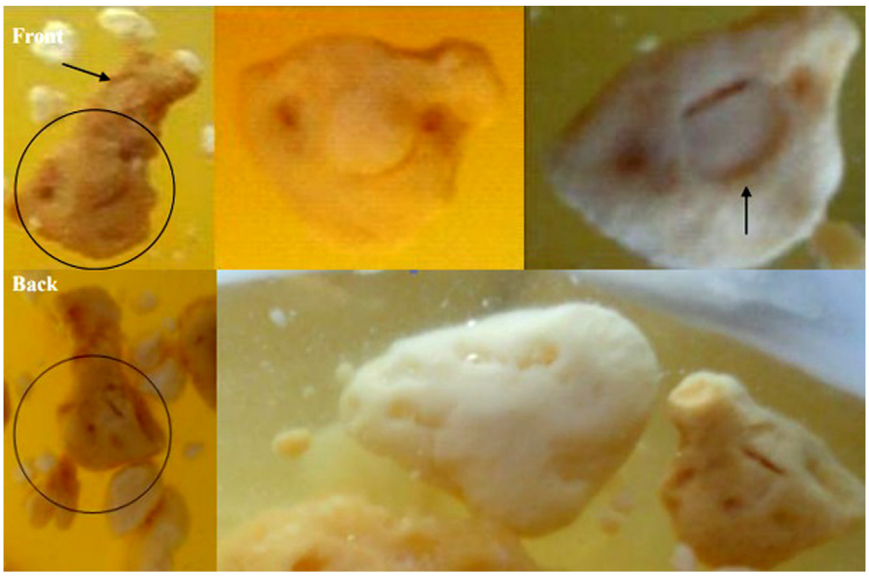

FIGURE 14.9 Another illustrative example of grain self-propagation. This is another documented example of sequential crack growth and splitting events. A smaller grain embedded in a larger grain (*circled in black, front and back*) was identifiable on either side. Because the grain has turned over, the other side has been exposed (*compare front and back*). Evidence of physical linkage between smaller and large grain is observable on the opposite side. Note the bud formation at the zone of splitting. The presence of pockmarks (*arrows*) suggest continuous release of globular clusters (assumed to represent nascent grains, ~2 to 3 mm). Starting grain measuring ~1 cm. Smaller grain ~0.7 cm. Time-series images over 3 weeks.

Tracking was difficult among visually similar grains, yet possible because most grains were distinct in a combination of features, i.e., the conjunction of color, form, and pattern that made them visually unique and easier to track (Fig. 14.11).

Behavioral data collected could tell how they interact with each other and their environment. During the search for certain behavior patterns, the author has witnessed unique and captivating views of the grains going under water, then coming back up, mutually engaged in some sort of "courtship" behavior. Remarkably, direct experimental evidence of pair formation was found no matter how crowded the culture had become. The grains appear to be engaged in some form of communication and there is a sense of close connection taking place (Figs. 14.12 and 14.13). A few times, they are so near each other that their surfaces almost touch.

Why do some grains appear to be attracted to other grains is not known. It is not within the scope of the report to discuss in details, the intricacies of symbiotic relationships likely occurring in this system. It is plausible that such apparent close connections occur through transmission of chemically complex molecules from one grain to the other, and/or signaling-based communication. References (Fuqua et al., 1996; Stadie, Gulitz, et al., 2013) provide excellent reviews of metabolic activity and symbiotic interaction of bacteria and yeasts. Bacterial species in particular can follow

FIGURE 14.10 Special grouping and motions captured in a series of panoramic views. When comparing the three-panel composite image, it is apparent that the relative orientation of any given grain with respect to each other is quite different. Within one minute, the grains have gotten closer to each other, changing position in response to invisible stimuli (*dotted lines*). There is a sense of close connection accentuated by their physical proximity. Within each group (*circled*), grains are seemingly engaged in "cross-talk." In the last image, one can see a pair of grains in close proximity (same pair also visible in the other images). Grains measured 0.5 and 1.5 cm.

gradients of nutrients and other environmental stimuli. Interactions may occur via chemotaxis and cell contact. Cell-to-cell communication is a complex phenomenon, and extensive research has been undergoing. Bacteria cells can communicate information about population density and metabolic state (called quorum sensing) and release signaling chemicals (Frey-Klett et al., 2011; Gobbetti & Di Cagno, 2013; Nadell et al., 2009; Salmond et al., 1995). Regardless of the mechanism involved, each grain presumably provides a continuum of microbial life forms able to survive as a close community by gaining support from each other. Survival depends on the many contributions of each grain within the culture.

14.4 Conclusions or future prospective

This report shows that KGs can reform from microbial isolates producing sustainable life forms. When maintained in an ECM system, smaller grains are released through cracks and splits from larger grains, and it was possible to also visualize their motion and engagement in dynamic grouping suggestive of pair-bonding behavior as never seen before.

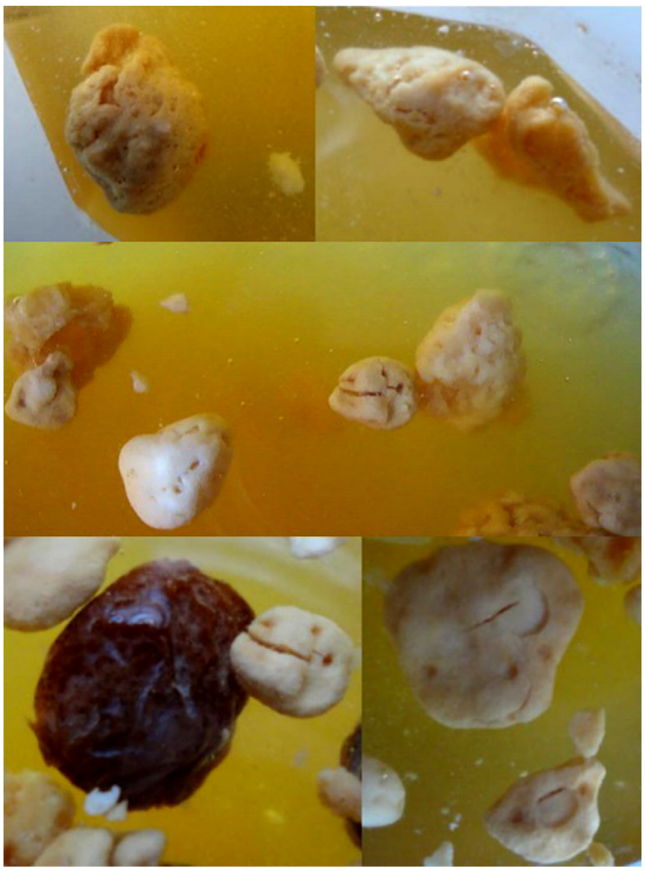

FIGURE 14.11 Grains seemingly living in a close community. The ECM system brought a variety of grains together. The grains are complex mixtures of microorganisms that seem to receive a significant advantage from interacting with each other, and various biofilm surfaces, the most important probably being to share resources, adapt and survive. Together, they continually explore the changing landscape around them, presumably in search of symbiotic relationships. They seem to be attracted by yeast-rich surfaces of food supplements. A plumped fruit (sun-dried date) has risen to the surface during active fermentation and floats right next to an aged grain. Images obtained on different days, when looking from over the culture and/or through the glass walls. *ECM*, experimental culture medium.

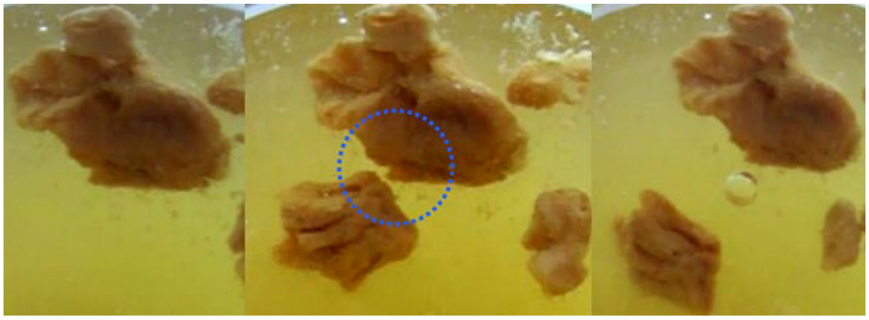

FIGURE 14.12 Example of pair-bonding behavior sustained during repeated fermentation cycles (Grains C and F from Fig. 14.4). There is a close connection visible with the naked eye (*represented by circles*). It is tempting to say that regardless of spatial location, some grains can locate other grains and interact specifically with them. The reader should refer to the reference material to aid in grain identification. Close views show grain F upside down with an extended flap of biomass.

The grains are living entities that congregate in pairs and/or larger groups. As part of a close community, they move in different directions and interact in unforeseen ways. It is likely that though not visible to the naked eye, free-moving grain observations from the ECM system also occur to a different degree in the traditional milk culture system.

The author invites the readers to experiment on KGs. There is still a lot more one can possibly learn about these grains. The ECM system has permitted an unprecedented, real-time view of self-propagation and pair-bonding behaviors. At present, the nature of these interactions is not understood. Studies would be required to understand how the grains relate to their surroundings, and to determine the way morphological changes can influence interactive behavior (s). This work raises many other interesting questions about how the grains communicate with each other, and can transform into sustainable life forms. In this regard, it may be interesting to characterize the diverse strains involved in this complex ecosystem.

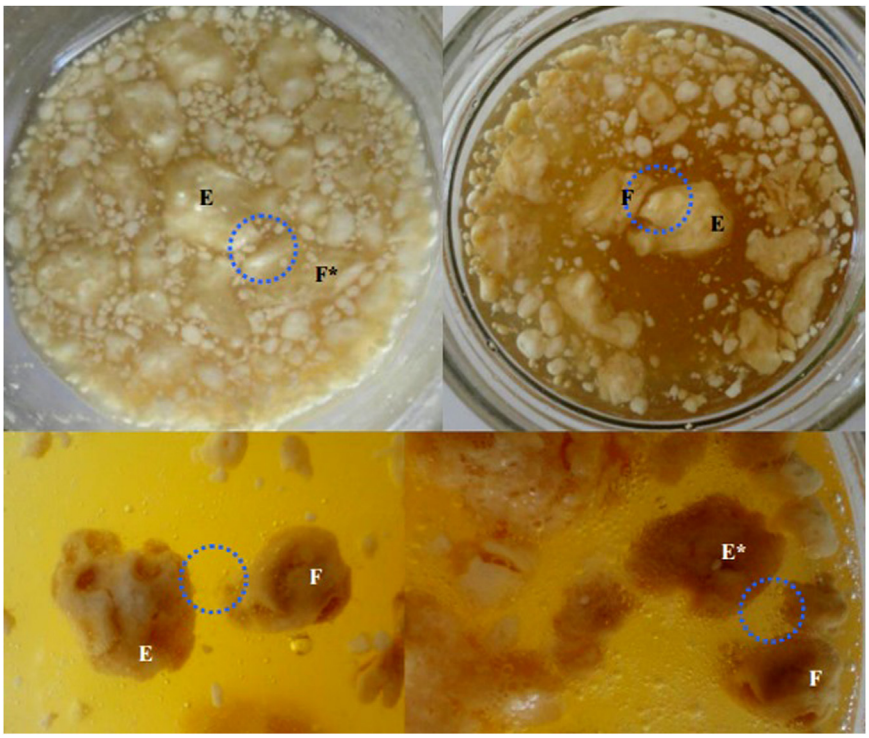

FIGURE 14.13 Another example of observable pair-bonding (Grains E and F from Fig. 14.4). This type of pair-bonding is not easy to track because grains can turn over, thereby exposing a different visual form (*). The grains are not easily recognizable since they do not hold the same shape and have a different orientation. The two grains have a tendency to cluster together. There is apparent visual clues of connectivity (*represented by circles*). The weekly recurrence of this pair-bonding phenomenon suggests that the grains did not interact randomly but are attracted to each other, even in crowded places.

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Chapter 15

Innovations in preservation and improving functional properties of kefir

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15.1 Introduction

In the past decades, eating habits and lifestyles have undergone several changes, which leads to an overload on the body's systems (Akkasheh et al., 2016; Andrade et al., 2020; Beltrán-Barrientos et al., 2016; Bian et al., 2018; Bourrie et al., 2016). There was an increase in the food supply, but a reduction in their nutritional quality was observed. Several factors could contribute to this reduction in nutritional quality, such as:

1. depletion of the concentration or availability of nutrients in the soil;
2. nutritional loss caused by improper storage, transport, and handling; and
3. loss of nutrients and chemical contamination caused by the industrialization of food, among others.

Simultaneously, the human body has changed, starting to require a more significant amount of nutrients to deal with the imbalances generated by environmental pollution, physical and emotional stress, and the increase in food consumption with antinutritional and industrialized factors. Functional clinical nutrition is a new way of science to approach nutrition, aiming to evaluate the organism's interaction with food. It is necessary to nourish the organism properly, in quantity and quality, to provide all the essential nutrients for its proper functioning and to ensure that these foods are well digested, absorbed, and used (Akkasheh et al., 2016; Beltrán-Barrientos et al., 2016; Bian et al., 2018; Bourrie et al., 2016).

The human microbiota, especially the intestinal microbiota, plays a protective role against pathogenic bacteria, preventing dysbiosis. Any change in the composition of the intestinal microbiota is called dysbiosis. Changes in its composition can lead to an increase in intestinal permeability, resulting in an increased lipopolysaccharides to the systemic circulation, which generates metabolic endotoxemia, and the development of a chronic inflammatory state. Dysbiosis causes symptoms such as gas, diarrhea, or constipation, and it is also related to cardiovascular diseases, metabolic syndromes, and disorders of the central and immune nervous system (Bercik et al., 2012; Betiku et al., 2018).

Probiotics are living microorganisms capable of preventing and controlling mainly gastrointestinal (GI) diseases, among other illnesses (Cheng et al., 2019; Kouchaki et al., 2017; Lee et al., 2018; Lorenzo et al., 2017; Mousavi et al., 2020; Pinto-Sanchez et al., 2017; Tian et al., 2020; Wang et al., 2020). Kefir is produced by the action of yeasts, lactic acid, and acetic acid bacteria that exist in symbiotic association in kefir grains (Enikeev, 2012; García-Burgos et al., 2020; Lopitz-Otsoa et al., 2006; Magalhães et al., 2011; Puerari et al., 2012; Rafie et al., 2015). The kefir's artisanal production is based on the tradition of Caucasus people, which has spread worldwide from the late 19th century, and nowadays integrates its nutritional and therapeutic indications to the everyday food choices of several populations (Enikeev, 2012; Lopitz-Otsoa et al., 2006). Many microorganisms present in kefir and their microbial interactions, the possible bioactive compounds resulting from microbial metabolism, and the benefits associated with this beverage gives kefir the status of a natural probiotic, designated as the 21st-century dairy beverage. Several studies also have shown that kefir and its constituents have antimicrobial, antitumor, anticarcinogenic, and immunomodulatory activity

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(Enikeev, 2012; García-Burgos et al., 2020; Lopitz-Otsoa et al., 2006; Magalhães et al., 2011; Puerari et al., 2012; Rafie et al., 2015; Tu et al., 2015).

The proper administration of probiotics, considering living organisms, tends to promote the human organism with numerous benefits, from the balance of the gut microbiota by gut-brain axis, to contributing to the increase of immunity, combating allergies, detoxifying the organism, regulating the organism to better absorb of nutrients and vitamins, among other benefits (Cheng et al., 2019; Kazemi et al., 2018; Kouchaki et al., 2017; Lee et al., 2018; Lorenzo et al., 2017; Marcial et al., 2017; Mousavi et al., 2020; Pinto-Sanchez et al., 2017; Tian et al., 2020; Wang et al., 2020).

This chapter includes the innovation aspects of the main beneficial effects of kefir for human health and in the preservation/improving its functional properties.

15.2 Historical report

Kefir grains originated in the Caucasus, and the origins of kefir grains predate known records. In ancient times, the Eastern nomadic shepherds discovered that the milk they were carrying in their leather purses during their travels sometimes turned into a foamy beverage. The nomads called the beverage "kefir." It is believed that the word originates from *keyif*, meaning "joy/pleasure" in Turkish (Enikeev, 2012). There is also another story in legends that the Prophet Muhammad gave the kefir grains to the people living in the northern Caucasian mountains, and kefir grains were then passed from generation to generation up to this day (Lopitz-Otsoa et al., 2006). Kefir is also known as kefyr, kephir, kefer, kiaphur, knapon, kepi, and kippi in different countries worldwide, wherever it is consumed (Enikeev, 2012; Lopitz-Otsoa et al., 2006).

15.3 Kefir: concept/characteristics, microbiology, and beverage preparation

Kefir is fermented milk and slightly effervescent. Fermented kefir food is easy to prepare and at low cost. Kefir has a natural probiotic microbiota present in the kefir grains (Magalhães et al., 2011; Magalhães-Guedes et al., 2019; Puerari et al., 2012; Rafie et al., 2015; Tu et al., 2015). Kefir grains are a symbiotic association of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria present in the kefiran polysaccharide matrix. Kefir grains have sizes between 0.5−3.5 cm in diameter, an irregular shape, and yellowish or whitish in color (Magalhães et al., 2011; Puerari et al., 2012; Rafie et al., 2015; Tu et al., 2015).

The microbial diversity and population of kefir grains vary according to the region of origin, the substrate used for the proliferation of grains, and the techniques used in their manufacture (García-Burgos et al., 2020; Magalhães et al., 2011; Puerari et al., 2012; Rafie et al., 2015; Tu et al., 2015). The main microbial genera found in kefir grains and beverages are shown in Fig. 15.1. The microbial genera are formed by species of LAB, acetic acid bacteria, and yeasts (Magalhães et al., 2011; Puerari et al., 2012).

Kefir grains are capable of fermenting on various substrates, such as cow, goat, sheep, and buffalo milks, brown sugar, fruit juices, and soy extract, among others (García-Burgos et al., 2020; Magalhães et al., 2011; Puerari et al., 2012; Rafie et al., 2015; Tu et al., 2015). The microbial species from kefir grains carry out three types of fermentation during the process: lactic, alcoholic, and acetic; kefir can quickly adapt to different substrates and lead to the production of new probiotic products (Magalhães et al., 2011; Puerari et al., 2012). The traditional way of kefir beverage production is using pasteurized or UHT (ultra-high-temperature processing) treated milk (Fig. 15.2). This kefir beverage is appropriate for consumption (Magalhães et al., 2011).

15.4 Kefir probiotic microorganisms in the gut-brain axis relationship

According to previous studies, the connection between gut balance and mental/functional well-being receive the name "gut-brain axis," which is a complex system that helps the body decrease stress\ anxiety levels (Enikeev, 2012; Lopitz-Otsoa et al., 2006; Petra et al., 2015; Ross, 2017; Wei et al., 2018) in addition to improving the host's immune system (Tian et al., 2020; Wei et al., 2018). This axis involves the gut's functions, with the brain providing good health to the host (Petra et al., 2015; Ross, 2017; Wei et al., 2018).

The gut and brain send and receive information through the neural pathways and the blood system (Petra et al., 2015; Ross, 2017; Wei et al., 2018). The gut-brain axis also influences the endocrine functions and nervous system, and the host's behavior (Petra et al., 2015; Ross, 2017; Wei et al., 2018). The gut-brain axis is regulated by the neural, hormonal, and immune systems (Tian et al., 2020; Wei et al., 2018). The functional regulation of the gut-brain axis is decreased with specific changes in the stress\anxiety levels (Petra et al., 2015; Ross, 2017; Wei et al., 2018).

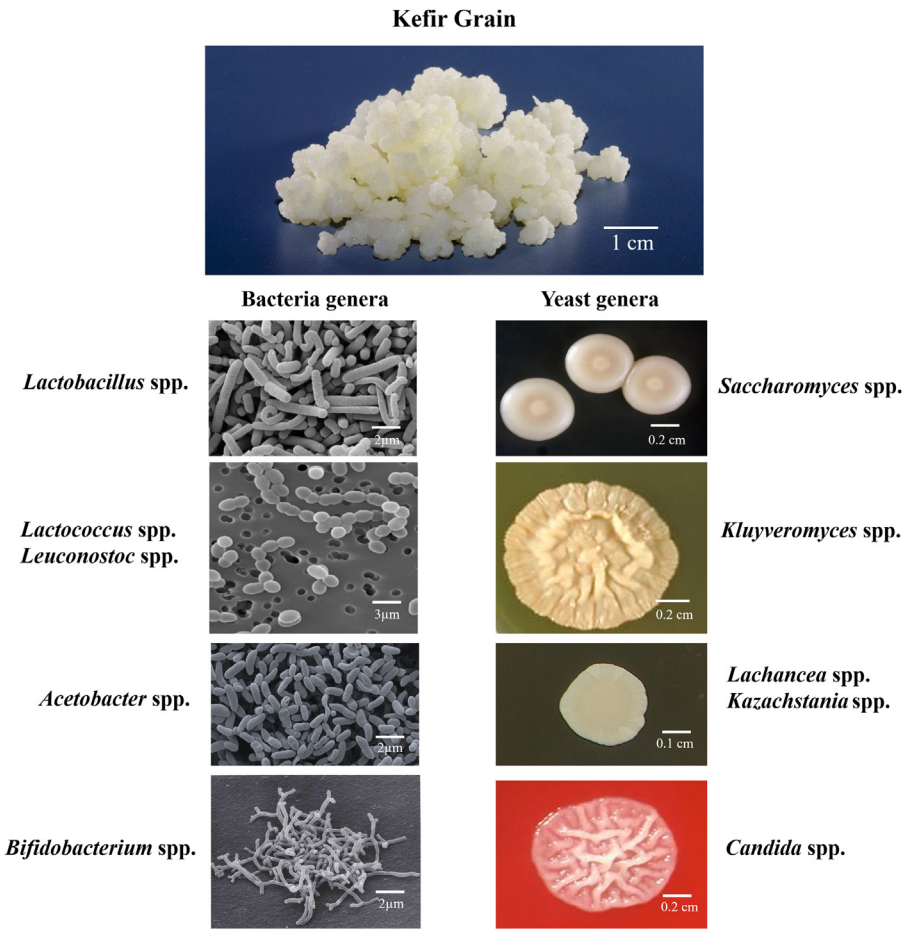

FIGURE 15.1 Microbial genera present in kefir grains and beverage.

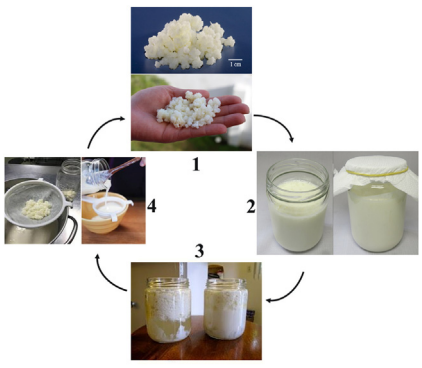

FIGURE 15.2 Milk kefir beverage production. Kefir grains (1) are added to milk and are left to stand at room temperature for fermentation 18–24 hours; (2) the milk is then fermented, forming the kefir beverage; (3) after which they are filtered; and (4) ready to start another cycle. The fermented milk that results from step 3 is appropriate for consumption.

The functional system of the gut-brain axis is represented in Fig. 15.3. Calcitonin gene-related peptide (CGRP) is a substance derived from calcitonin peptides. CGRP is produced by neurons and released in the intestine when the gut microbiota is impaired with an increase in pathogenic microorganisms (Wei et al., 2020). After the infection is activated and the CGRP secreted, the host defense and immune response are activated by the calcitonin receptor. When the gut microbiota is balanced by probiotic microorganism consumption, the CGRP has a regulatory effect on the gut-brain axis (Wei et al., 2020). Corticotropin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) are released on the hypothalamic-pituitary-adrenal axis and respond to the inflammation caused, in addition to activating the immune system against pathogens (bacteria, fungi, and viruses) (Wei et al., 2020).

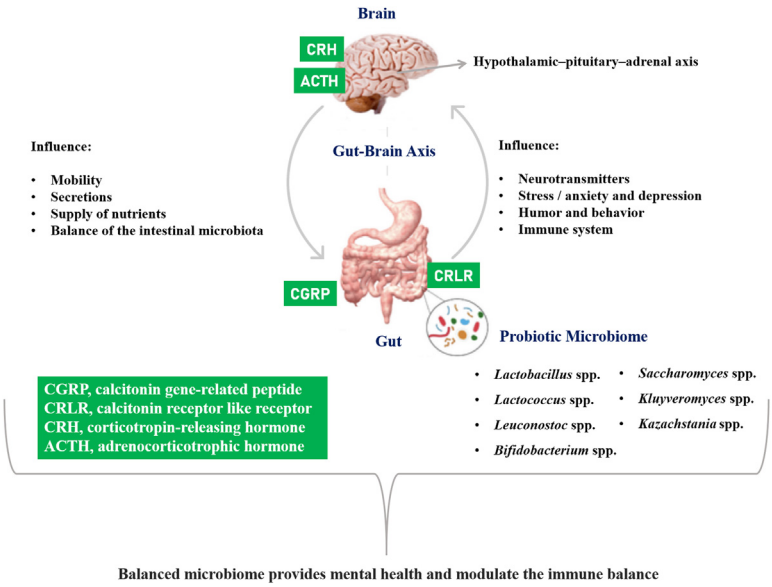

FIGURE 15.3 Functional system of the "gut-brain axis."

The hypothalamic−pituitary−adrenal axis is studied in humans and animals. Current studies examined the CRH administration in humans that reproduced neurological stress as CRH increased the gut permeability and the ACTH volume in plasma (Vanuytsel et al., 2014). In studies with mice infected with pathogenic microorganisms, they evaluated the action of the hypothalamic−pituitary−adrenal axis regarding CRH and ACTH activity. The mice showed anxiety and stress disorders, proving that the gut microbiota imbalance interferes with the hypothalamic−pituitary−adrenal axis (Vanuytsel et al., 2014).

The hypothalamic−pituitary−adrenal axis (HPA axis or HTPA axis) is a complex set of direct influences and feedback interactions among three components: the hypothalamus: the pituitary gland (a pear-shaped structure located below the thalamus) and the two adrenal (also called "suprarenal") glands (small, conical organs on top of the kidneys). These organs and their interactions constitute the HPA axis, a primary neuroendocrine system that controls reactions to stress and regulates many body processes, including digestion, the immune system, mood/emotions, sexuality, and energy storage and expenditure. It is the standard mechanism for interactions among glands, hormones, and parts of the midbrain that mediate the gut-brain axis (Vanuytsel et al., 2014). The gut-brain axis provides a host body communication that can cause several pathological consequences if there is functional deregulation (Bercik et al., 2012; Majeed et al., 2018; Marcial et al., 2017; Ross, 2017; Sarkar et al., 2016; Wei et al., 2020).

15.5 Functional properties of kefir

15.5.1 Kefir probiotic microorganisms in the immunomodulatory activity

To display a beneficial impact on the host health, probiotic microorganisms, including those present in kefir, should survive in the stressful/harsh conditions of the host's stomach and GI tract. Probiotic microorganisms promote host health by inhibiting pathogenic strains (Enikeev, 2012; Lopitz-Otsoa et al., 2006; Petra et al., 2015; Ross, 2017; Wei et al., 2018). Probiotic microorganisms compete for nutrients for growth and proliferation in the host gut, eliminating pathogens (Ross, 2017; Wang et al., 2020). Besides, probiotic microorganisms can influence the host's immune cell activity, regulating inflammation, barrier function, and cell-to-cell signaling (Wang et al., 2020). One way to foster healthy gut microbiota is to eat more natural and nutritious food and probiotic microorganisms. Increasing the intake of plant fibers, vegetables, fruits, legumes, whole grains, nuts, and seeds provides adequate nutrition for the gut's probiotic microbiota (Wang et al., 2020). The consumption of beneficial microorganisms (probiotics) and prebiotics, which includes the symbiotic foods, such as yogurt (Mousavi et al., 2020), kefir (Magalhães et al., 2011; Puerari et al., 2012), and kombucha, can promote a healthy gut (Andrade et al., 2020).

Studies on the probiotic microorganism's actions on the host immune system have been carried out. Non-recent and recent bibliographies, most reporting studies with LAB, showed that probiotics have an immune-stimulating effect in

animals and human, although the mechanisms by which this occurs are not yet fully understood (Ross, 2017; Wang et al., 2020).

The immune-stimulating effect may be related to the probiotic microorganism's ability to interact with Peyer plaques and intestinal epithelial cells, stimulating IgA-producing B cells and T-cell migration from the gut (Wang et al., 2020). Regular use of probiotics increases the macrophages phagocytic activity, stimulating the immune system (Wang et al., 2020). The immune system of the body must be able to identify pathogens organisms to fight the infections. These organisms have antigen molecules on their surface. The immune system reacts to the pathogen presence in two ways: (1) The innate immune response is a rapid reaction. Innate immune cells recognize the antigens found on pathogens. These cells react to signaling molecules released by the body in response to infection. Innate immune cells quickly begin fighting an infection and result in inflammation. The cells involved in this reaction can kill pathogens or activate cells involved in adaptive immunity. (2) The adaptive immune response was slower than the innate response but can target specific pathogens better. T and B cells are the primary cells involved in this response, killing pathogens and infected cells. Other T cells help control the adaptive immune response. The primary function of B cells is to make antibodies against specific antigens. Antibodies, also known as immunoglobulins, are proteins that attach themselves to pathogens. These signal immune cells to destroy the pathogen. Some T and B cells change into memory cells to combat reinfection by the same pathogen (Wang et al., 2020).

Different *Lactobacillus* strains are consumed. Lactobacilli can be used in immunotherapeutic applications such as an "oral vaccination−therapy" for T-cell tolerance induction against autoimmune disease (Maassen et al., 2000). Oral administration of *Lactobacillus reuteri* and *Lactobacillus brevis* induced expression of the pro-inflammatory cytokines (Maassen et al., 2000). Cytokines are small proteins ($\sim$5−20 kDa) essential in cell signaling. Cytokines modulate the balance between humoral and cell-based immune responses and are essential in health and disease, specifically in host immune responses to infection and inflammation (Maassen et al., 2000). A selection of specific strains of *Lactobacillus* provides a strategy to influence cytokine expression and generate an immune response, improving the host immune system (Maassen et al., 2000).

Lactobacillus rhamnosus reduced the cell multiplication of *Escherichia coli* O157: H7 in infected mice experimentally, stimulating the macrophage activity and IgA antibody (Shu & Gill, 2002). Shu et al. (2001) reported that piglets treated with *Lactobacillus lactis* HN109 showed decreased diarrhea associated with *Rotavirus*, increasing antibodies against specific pathogens in the GI tract. Treatment also increased the concentration of blood neutrophils and the proliferative response of T lymphocytes.

15.5.2 Kefir probiotic microorganisms in antitumor anticarcinogenic activity

The anticarcinogenic definition is "tending to inhibit or prevent the activity of a carcinogen or the development of carcinoma," according to *Merriam Webster Medical Dictionary*. Tumors are classified as carcinomas and sarcomas (John & Deeseenthum, 2015). There is an interest in the antitumor and anticarcinogenic benefits of kefir consumption (dos Reis et al., 2019; Liu et al., 2002; Shiomi et al., 1982).

Studies have been conducted with experimental mice or in vitro, indicating some success in reducing tumor size with kefir beverage and grains. In the first report, an investigation of the antitumor effects of a water-soluble polysaccharide isolated from kefir grains indicated that the polysaccharide was able to inhibit the growth of Ehrlich carcinoma compared to the control mice (Shiomi et al., 1982). Furukawa et al. (1990) indicated that feeding with kefir grain (2 g/kg body weight) was more effective in inhibiting tumors (Lewis lung carcinoma) when given for 9 days after tumor inoculation to the mice. Another study focused on the effects of oral administration of cow milk and kefir beverages (soy and milk) on tumor growth in mice inoculated with sarcoma-180 tumor cells that resulted in $\sim$65% and $\sim$71% inhibition of tumor growth, respectively, compared with controls (Liu et al., 2002).

dos Reis et al. (2019) evaluated the effect of regular milk kefir beverages on preneoplastic colonic lesions. Thirty Wistar rats received water (control group) or milk (milk group) or kefir beverage (kefir group) for 5 weeks. After that, colonic lesions were chemically induced, and the treatments continued for more than 13 weeks. The regular consumption of kefir beverages reduced the incidence of aberrant crypt foci by 36%. Also, kefir beverage consumption increased the cecal concentration of short-chain fatty acids, promoted the colonic concentration of TNF-α and IL-1β, and reduced the lactulose/mannitol ratio and the enzyme catalase in comparison with the control group. Therefore, milk kefir beverages reduced lesion development, probably by increasing short-chain fatty acids, reducing intestinal permeability, immunomodulation, and improvement of colonic antioxidant activity.

15.5.3 Kefir probiotic microorganisms in antimicrobial activity

The main probiotic effect of kefir is modulating the host's intestinal microbiota, where there is a reduction in undesirable microorganisms and an increase in the number of beneficial microorganisms, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Enikeev, 2012; Lopitz-Otsoa et al., 2006; Petra et al., 2015; Rattray & O'Connell, 2011; Ross, 2017; Wei et al., 2018). The antimicrobial substances of kefir, such as lactic acid, acetic acid, and H_2O_2, have been reported to have bactericidal and bacteriostatic effects on bacterial pathogens (Karatepe & Yalçın, 2014). Lactic acid reduces the GI tract's pH, which is not suitable for the growth of pathogens. Besides, H_2O_2 has an antagonistic and acetic acid has an antibacterial effect on pathogens (Rattray & O'Connell, 2011). *Salmonella* spp., *Yersinia* spp., *Shigella* spp., *Micrococcus* spp., *E. coli, Bacillus cereus*, *Candida albicans*, and *Klebsiella pneumoniae* are some of the pathogens microorganisms inhibited by kefir (Rattray & O'Connell, 2011). The research focused on the undesirable microbial inhibition activity of kefir, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, and *E. coli* were used as the target microorganisms, which are known to promote the GI diseases such as diarrhea. According to the research results, kefir showed the microbial inhibition activity against all of the target microorganisms (Sirirat & Jelena, 2010).

Many other studies showed the antibacterial activity of kefir and kefir grains against various pathogen bacteria and yeasts such as *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Streptococcus* pp., *Yersinia* spp. and *Micrococcus* spp. (Cevikbas et al., 1994; Garrote et al., 2000; Gulmez & Guven, 2003; Rodrigues et al., 2005; Yüksekdağ et al., 2004).

15.6 Preservation and improving functional properties of kefir

In this topic, some scientific studies on preserving and improving kefir's functional properties will be addressed. This fact is essential for better use of the potentially functional characteristics present in kefir:

The following shows, from one study, the impact of preservation and different packaging conditions on the microbial community and activity of kefir grains (Witthuhn et al., 2005). The study evaluated three different packaging materials in their ability to retain the viability and activity of the kefir grains over an extended storage period. Kefir grains were lyophilized, packaged in three different packaging materials, including low-density polyethylene film (LDPE), oriented polyester film (OPET), and metalized oriented polyester film (MOPET), and stored for 3 months at room temperature. Activity tests, including pH, titratable acidity (%TA), lactose, and lactic acid content over a 10−18 hour fermentation period, were used to evaluate the acidification activity of the lyophilized grains. Selective media, morphology, and physiological characteristics were used to obtain the enumeration values and identify the microbes present in the packaged and stored grains. Overall, the best retention of the fermentation activity was found for the MOPET film. The OPET packaging film provided the best preservation of the microbial composition. Based on the data obtained from this study, it was concluded that there were differences between the three different packaging materials in terms of their ability to preserve the acidification activity of the lyophilized kefir grains. The MOPET film preserved the microbial acidification activity better than the LDPE and OPET films, possibly since it is a better oxygen and moisture barrier. The storage period of 3 months had a considerable negative influence on the lyophilized grain's activity, resulting in decreased activity and prolonged lag phases of the kefir grains stored for 3 months. In this study, the data clearly showed that lyophilization and packaging of kefir grains resulted in a reduction of the microbial numbers and the number of microbial species, compared to kefir grains that were not lyophilized. Although the microbes isolated from the kefir grains packaged in LDPE, OPET, and MOPET did not vary from each other, the microbial species isolated from the packaged kefir grains were very different from the microbial species isolated from kefir grains that were not lyophilized and packaged. Therefore, it is clear that the lyophilization and packaging of kefir grains do have an impact on the microbial community of kefir grains.

Optimization of the spray drying process parameters for kefir powder using response surface methodology (RSM) (Atalar & Dervisoglu, 2015) include RSM used to optimize the spray drying conditions to produce kefir powder. Influence of inlet air temperature (120°C−180°C), feed temperature (4°C−30°C), and pump rate (20°C−40%) on the survival rates of microorganisms, outlet temperature, moisture content, and water activity were assessed after drying and modeling by RSM. Inlet temperature was found as the main factor that affects all responses statistically significant ($P < .05$). The effect of the pump rate on the responses was found significant for some. Feed temperature has no significant effect on any response. The optimum conditions were at 135°C inlet air temperature and 35% pump rate using the kefir biomass digestion function. The optimum conditions of spray drying were matched with freeze-drying results,

showing good quality powder. Kefir powder can easily be used when it is desired. These advantages of kefir powder can increase the consumption of kefir in the homes.

The suitability of kefir powder production using spray drying (Teijeiro et al., 2018) including the survival of microorganisms after drying, storage, and simulated GI conditions was investigated. Milk and whey permeate were used as a substrate for kefir growth and were dehydrated directly (traditional kefir) or with different carriers (skim milk, whey permeate, and maltodextrin). Kefir grains dried without carriers showed low microbial survival (5.5 log and <2 log CFU/g for LAB and yeast, respectively). However, the survival of the microorganisms was significantly improved in the presence of different carriers. The LAB population was above 9 log CFU/g using skim milk (SM) as a carrier, but yeast viability was dramatically reduced after spray drying. When whey permeate was used as the carrier medium, LAB survival was 8 log CFU/g, and yeast survival was above 4 log CFU/g. LAB in the kefir powder survived better in the simulated GI conditions when spray drying was conducted in SM. LAB in a kefir powder sample dehydrated in SM and SM plus maltodextrin remained stable for at least 60 days at 4°C. The results demonstrated that spray drying is a suitable approach to obtain a concentrated kefir-derived product. Spray drying of kefir leads to many advantages for storage and transportation. The study demonstrated that selecting suitable carriers for the spray drying process is crucial for the viability of kefir microorganisms during both the drying process and storage. Even the viability of the more susceptible microorganisms present in kefir, yeasts, was improved when appropriate. These findings support spray drying as a potential method for obtaining dehydrated products derived from kefir.

There is an alternative way to encapsulate probiotics within electrospun alginate nanofibers as monitored under simulated GI conditions and in kefir (Yilmaz et al., 2020). In this study, *Lactobacillus paracasei* KS-199 was encapsulated within alginate-based electrospun nanofiber mats possessing uniform, well-defined, smooth and bead-free structure with an average size of 305 nm in diameter. The mats' enhanced protection ability was observed in thermal degradation assays (weight loss from 93.4%−84.5%). Probiotic and in situ viabilities were determined in simulated GI and in vitro conditions, respectively, demonstrating that nanoencapsulation of the strain could enhance its survival in simulated gastric juice (the viability rate from 64.1−70.8 log cfu/mL) and improved its viability/survival (from 6.65−7.38 log cfu/mL) in kefir, respectively. Inoculation of kefir with the encapsulated strain did not change the characteristic pseudo-plastic flow behavior and viscoelastic nature (being predominantly elastic than viscous; $G' > G''$ values) of kefir. It was suggested that alginate could be an essential tailor-made biopolymer playing an essential role in the probiotics' encapsulation with increased viability. The nanofiber diameter, zeta potential, molecular, thermal, and morphological analysis results in this study confirmed that living cells of *L. paracasei* KS-199 could be accomplished encapsulated within the alginate-based electrospun nanofiber mats. Moreover, the fabricated nanofiber mats had a uniform, well-defined, smooth, and bead-free structure with an average of 305 nm in diameter. However, encapsulation of *L. paracasei* KS-199 within the spin solutions yielded in beaded fiber structure with an average size of 842 nm for strain-loaded nanofiber mats, revealing the successful encapsulation of the probiotic strain. The nanofiber mats had a high melting temperature, implicating their future utilization in the heat-processed and bakery foods where they should maintain its stability at high processing temperature levels ($\sim$220°C or above). The results confirmed the importance of this temperature of the encapsulation matrix to determine encapsulated probiotic bacteria's survival. The loaded cells could retain survival/viability even after being exposed to high voltage levels and storage under refrigerated conditions, implicating that the electrospinning process did not remarkably influence the stability and metabolism of *L. paracasei*. Nanoencapsulation could also improve the survival of the strain in simulated gastric juice, making it possible to expect an enhanced survival of this strain during their passage through the stomach and then facilitate its colonization. The in situ viability test results also showed that nanoencapsulation of *L. paracasei* into nanofiber mats improved its viability in kefir, revealing that alginate as a biofunctional polymer could effectively maintain the survival of *L. paracasei* KS-199 during gastric digestion. On the other hand, kefir's inoculation with an -encapsulated strain did not change the characteristic pseudo-plastic flow behavior and viscoelastic nature of kefir. The results of this work showed that electrospinning is an excellent technique to fabricate alginate-based nanofibers for encapsulation of the probiotic strain by keeping them intact. The increased viability of probiotics is significant for the functional food industry to develop functional food products with enhanced bioactive properties. The results suggested that the alginate-based electrospun nanofiber mats showed great potential for the enhanced protection and delivery of the *L. paracasei* in food products, making them a novel probiotic carrier development of functional foods. The results also revealed the improved performance of alginate for encapsulation of probiotic strains in the production of even heat-processed foods. These results revealed that alginate could be a critical tailor-made biopolymer playing an essential role in successfully encapsulating probiotics with increased viability. Thus, the alginate-based nanofiber mats could be employed to develop novel functional foods, having enhanced bioactive properties as quantified by thermal degradation and in vitro assays.

15.7 Conclusion and future potential

An increasing number of reports have emerged and provided evidence for the effects of probiotics microorganisms on the gut-brain axis's balancing act. Some of the single or multiple microbial strains can beneficially affect the central nervous system functions mediated by the gut-brain axis, improving the host immune system, including mood, anxiety, depression, and stress—the results found are good host defenses against microbial pathogens.

For centuries, kefir has been known as a much more complex probiotic than the other probiotic products as it has a unique chemical and microbiological composition. Many scientific studies indicate that kefir has various functional properties such as antimicrobial, antitumor, anticarcinogenic, and immunomodulatory activity. This magical fermented dairy beverage appears to have great potential, and other laboratory and clinical studies should be carried out on kefir to analyze the hidden therapeutic and functional properties that have not been noticed yet.

A suggestion for future studies is to investigate detailed descriptions of the kefir probiotic microorganisms, including genus, species, and strain daily dose and duration used so that the appropriate data can be grouped and analyzed. Metabolomics can be easily applied to identify kefir's detailed composition and record the biochemical changes due to microbial activity during the fermentation and storage process. Metabolomics is the scientific study of chemical processes involving metabolites, small molecule substrates, intermediates, and metabolism products. Specifically, metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind, studying their small-molecule metabolite profiles. Besides, scientific studies on the preservation and improving functional properties of kefir must be carried out. This fact is essential for better use of the potentially functional characteristics present in kefir.

The beneficial effects of kefir on the intestine and mucosa should be elucidated to understand better the gut mucosa's immunological maturation and dairy consumption. Kefir is a crucial opportunity for the dairy sector.

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Further reading

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Chapter 16

Health benefits of probiotics: an overview

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16.1 Introduction

The public acceptance and understanding about probiotics are increasing, and industry of probiotics is growing at 7% annually (Cunningham et al., 2021). Roobab et al. (2020) reported that nutritionists and gastroenterologists commonly recommend the use of probiotics, the former for prevention of disease and maintenance of good health, while the latter for treating diseases in the gastrointestinal (GI) tract. Draper et al. (2017) reported that most of the health care providers (61%) routinely prescribed medication, but also probiotic supplements or foods to their patients.

The International Scientific Association for Probiotics and Prebiotics have promoted panels wherein professionals reviewed and posted the technological know-how in the back of probiotic cultures. They concluded that probiotics may play an important role in health status (Cunningham et al., 2021). Probiotics are defined as live microorganisms, that when administered in suitable amounts, confer a health benefit on the host. In this way, probiotic cultures, at suitable counts, can modify positively the microbiota, making it healthier and generating eubiosis (López-Moreno et al., 2020). *Bifidobacteria* and *Lactobacillus* are commonly used probiotic cultures.

Zheng et al. (2020) presented the formal division of the genus *Lactobacillus*, with about 260 species, in 25 genera. According to Pot (2020), the different clusters obtained were shown to be intertwined with species from other genera such as *Fructobacillus*, *Leuconostoc*, *Paralactobacillus* and *Pediococcus*. The original *Lactobacillus* genus, defined in 1901 around the species of *Lactobacillus delbrueckii*, is now again restricted to only 35 species. The other species were formally removed from this genus and allocated to 23 other new genera and to the genus *Paralactobacillus*. The positive aspect of this major reclassification, however, is that only the name of the genre was changed. The species names remain the same. In addition, the authors were concerned with trying to choose names for new genres that also started, for the most part, with "L," which means that in short, not everyone was modified. Unfortunately, not all genres start with "L."

There are different mechanisms of action used by probiotic strains that are dependent on the strain. Some of the mechanisms of action have already been discussed, but structure–function explanations and long-term influences should be better discussed. Probiotics interchange with both the microbiome and the host by molecular effectors secreted (metabolic products) or included in the cell structure. The former can modify the microbiota by changing the microenvironment in the gastrointestinal tract (decreases in pH values), competing with pathogens for binding sites or nutrients, interacting by cross feeding, and producing bacteriocins and/or antibacterial compounds. The effects on the microbiota suggest the possible use of probiotics to decrease the pathogen growth and ameliorate oral and vaginal dysbiosis. Probiotics may interact with receptors in enteroendocrine, intestinal, immune, and epithelial cells and vagal afferent fibers, with improvements in the intestinal barrier integrity and inflammation (Toll-like receptors). Furthermore, systemic effects in host immune, nervous, and endocrine systems mediators can be observed. Probiotics can also metabolize compounds of the host, such as xenobiotics and bile salts. Specific probiotic effector molecules

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associated with the surface, such as lipoteichoic acids, pili, exopolysaccharides, and various surface-layer proteins can result in important effects, which are strain specific (Cunningham et al., 2021).

Probiotic cultures can inhibit foodborne and gastroenteric pathogens and fungi associated with food spoilage, mainly, because they can survive to the GI conditions, form biofilms, and adhere to the layer of mucin. *Bacillus subtilis* and *Bacillus coagulans* may have antisclerosis activity, *Saccharomyces cerevisiae* may have antiulcerogenic activity, and *Lactobacillus* spp. may have antimicrobial activity against *Clostridium difficile* and *Salmonella enterica*. The consumption of probiotics or synbiotics could decrease inflammation associated with arthritis, inflammatory bowel disease, fatty liver, among others (Roobab et al., 2020). However, more clinical trials are needed to study the gut microbiome and evaluate if the results could be generalized to all people. Additionally, the daily ingestion of probiotic yogurt (*B. lactis* and *Lactobacillus acidophilus*) could reduce the blood sugar and improve the endothelial function, resulting in reduced risk of cardiovascular disease (Roobab et al., 2020).

This chapter aims to present data from scientific articles on the effects of probiotics on human health with an emphasis on obesity, diseases of the respiratory tract and intestine-brain axis and animal health and the challenges to be faced in the next years. Furthermore, the impact of probiotic administration on the reduction of health care costs is discussed.

16.2 Probiotics and the obesity

More than 1.9 billion adults older than 18 years were overweight in 2016, representing 39% of the adults (40% women and 39% men). Furthermore, there were 650 million obese adults. Overall, part of the world's adult population (26%, being 15% women, 11% men) were obese. Obesity has increased three times worldwide between 1975 and 2016 (WHO, 2020), and reached warning proportions (Borgeraas et al., 2018).

More than 38.2 million children younger than 5 years were overweight or obese in 2019. Overweight and obesity has been considered a problem in high-income countries; however, it is now increasing in low- and middle-income countries, mainly in urban settings. In Africa, the quantity of overweight children (younger than 5 years) has increased about 24% since 2000. In Asia, about 50% of the children younger than 5 years were obese or overweight in 2019. Furthermore, at least 340 million children and adolescents (5−19 years old) were obese or overweight in 2016, and the number has increased from about 4% in 1975 to over 18% in 2016. There were no gender differences, with 18% girls and 19% boys who were overweight in 2016. Obesity and overweight have been responsible for more deaths than underweight, as there are more obese people than underweight, excepted in Asia and sub-Saharan Africa (WHO, 2020).

Overweight and obesity are chronic diseases widespread around the world. They are associated with an excessive intake of calories and insufficient expenditure of energy, resulting in a higher risk of several chronic diseases, such as hyperlipidemia, type 2 diabetes, hypertension, and cancer. Overweight/obesity, which is associated with dyslipidemia and loss of glycemic control, could seriously affect the patients' quality of life and increase their economic burden. The prevalence of obesity was associated with a gut microbial dysbiosis, inducing alteration of energy absorption by the host and influencing the fasting-induced adipose factor and the intestinal permeability (Wang et al., 2019).

The supplementation of diet with probiotics can promote the modulation of the gut microbiota dysbiosis and improve and/or prevent metabolic diseases, such as diabetes, obesity, metabolic syndrome, and other comorbidities. The consumption of probiotic formulas was safe and could modify clinical biomarkers. However, unspecific or discrete outcomes have been reported for consumption of probiotics and obesity, with scarce data concerning the modulation of human microbiota, making difficult to establish a protocol and make health assumptions. The probiotic health effects have been demonstrated by the scientific evidence and the safety history, such as those for commercial multispecies VSL3, and for mono-strains, such as *Lactobacillus rhamnosus* GG, *L. casei* Shirota, and *Bifidobacterium breve B-3* (López-Moreno et al., 2020).

Fig. 16.1 presents the main mechanisms of action for probiotics on obesity/overweight. The metaanalysis and systematic review performed by Wang et al. (2019) examined the effects of diet supplementation with probiotics in overweight and/or obese healthy adults. The results suggest that probiotics may contribute to weight loss and improve the lipid profile and glucose metabolism. They concluded that the supplementation with probiotics could reduce the fat mass and body weight and improve some of the lipid and glucose metabolism parameters, although some of the effects were small. The authors also highlighted that probiotics could be considered a new tool for the prevention and/or treatment of obesity and overweight in adult individuals.

The metaanalysis and systematic review published by López-Moreno et al. (2020) verified the most common *Lactobacillus* and *Bifidobacterium* species applied in disorders related to obesity and using clinical trials with humans, which were: *L. delbruecki*, *L. plantarum*, *L. casei*, *Streptococcus*, *L. acidophilus*, *L. paracasei*, *Pediococcus*

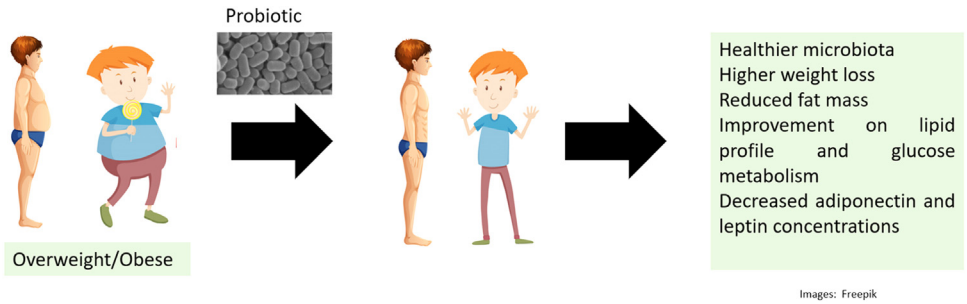

FIGURE 16.1 Mechanisms of action of probiotics on overweight/obese individual.

pentosaceus, *L. rhamnosus*, *Lactococcus lactis*, *L. gasseri*, *L. brevi*, *L. curvatus*, *B. longum*, *L. reuteri*, *B. infantis*, *B. breve*, *B. bifidum*, *B. animalis*, and *B. lactis*. They also highlighted the most common species of *Lactobacillus, Bifidobacterium*, and next-generation probiotics used in disorders related to obesity and using clinical studies with animals, which were: *L. plantarum, L. curvatus, L. rhamnosus, L. casei, L. acidophilus, L. paracasei, Streptococcus thermophilus*, *L. delbruecki*, *B. animalis*, *L. gasseri*, *B. breve*, *B. longum*, *B. bifidum*, *B. infantis*, *Hafnia alvei*, and *Bacteroides uniformis*. This study reported that probiotics can slightly improve the lipid metabolism, mainly by increasing the HLD-cholesterol levels and decreasing the total cholesterol. The TAG levels and LDL-cholesterol were not modified by the supplementation with probiotics. Furthermore, this study showed that the placebo group favored the CRP (C-reative protein) and glucose levels, which are parameters negatively correlated with symptoms of obesity and inflammatory diseases. The adiponectin (regulates lipid and glucose metabolisms) and leptin (regulates energy expenditure and food intake) are obesity-related hormones and may have a small but significant effect to decrease body weight and fat mass. This study also found that adiponectin and leptin concentrations were decreased after probiotic supplementation, however the results were correlated to lipid or glucose metabolisms and were associated with modification in the body weight (BMI) by the probiotics (López-Moreno et al., 2020). Borgeraas et al. (2018) selected 13 studies (from 800 at the beginning of the search) in their metaanalysis, which examined the effects of ingestion of probiotics on the body weight. They revealed that probiotic ingestion resulted in higher weight loss than placebo, and the studies had moderate heterogeneity.

Dairy product consumption has shown an inverse correlation with T2DM (diabetes mellitus type 2) risk, mainly low-fat milk and dairy products. Furthermore, an inverse correlation was presented after consumption of fermented dairy products, such as fermented milk and yogurts, which did not show a negative impact in a healthy diet considering the glucose metabolism. Milk may present oligosaccharides, which can act as prebiotic components for obesity and T2DM management. Dysbiosis of gut microbiota is related to T2DM, impacting the energy metabolism, and resulting in adiposity, and increased oxidative stress and systemic inflammation (Zepeda-Hernández et al., 2021). Thus, the modulation of diversity and the community of the gut microbiota by administrating probiotics can provide a healthier gut microbiota and improve the homeostasis, improving the management of chronical diseases, such as obesity.

16.3 Probiotics and respiratory tract diseases

Respiratory tract infections (RTI) are common infectious diseases of viral or bacterial origin. RTIs are commonly caused by virus, such as respiratory syncytial virus, rhinovirus, human parainfluenza virus, influenza virus, measles, human metapneumovirus, mumps, coronavirus, and adenovirus. RTIs caused by bacteria are less common, but they can be related to sporadic cases of respiratory diseases and outbreaks, including *Mycoplasma pneumoniae, Streptococcus pneumoniae, Coxiella burnetiid, Legionella pneumophila, Haemophilus influenzae*, and *Chlamydia pneumoniae*. Furthermore, bacterial bronchitis, sinusitis, and pneumonia can occur after a viral infection, resulting in a secondary infection (Darbandi et al., 2021).

Acute viral RTIs can increase substantially the health care resources and impact directly in the society. The administration of probiotics has been associated with reduced incidence and duration of RTIs (Lenoir-Wijnkoop et al., 2019). Fig. 16.2 presents the mechanisms of action for probiotics on RTIs.

Researchers of the York Health Economics Consortium (YHEC) and the Dairy and Food Culture Technologies carried out a metaanalysis and systematic review considering the effect of probiotic administration on the duration of acute respiratory infections in healthy adults and children (King et al., 2014). The authors reported that probiotic cultures may reduce the duration of the RTI episode. This study also verified fewer days of illness per person, shorter illness episodes and fewer days of absenteeism in participants who took probiotics compared with those who received a

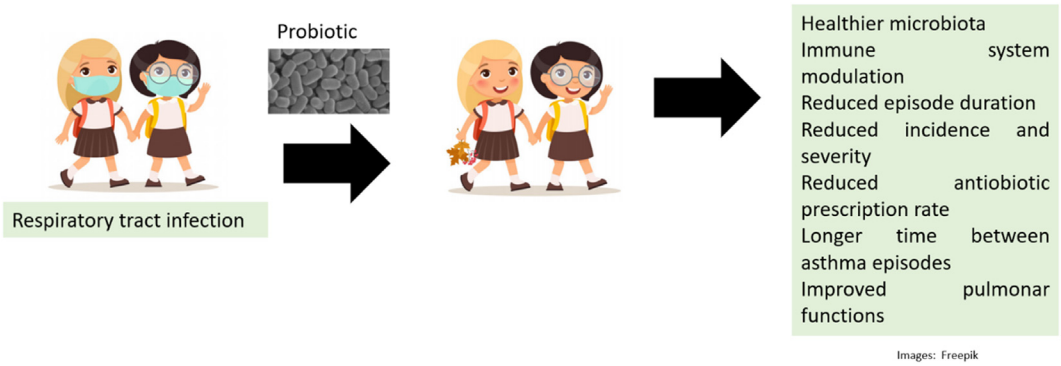

FIGURE 16.2 Mechanisms of action for probiotics on respiratory tract infection.

placebo. Hao et al. (2015) published at the Cochrane Collaboration an intervention review where the administration of probiotics and placebo were compared in healthy people of all ages in the prevention of acute RTIs; the comparison showed that probiotics reduced the incidence of RTI and the prescription rate of antibiotics.

According to Baud et al. (2020), the ingestion of probiotic cultures can decrease the severity and incidence of RTIs caused by viruses. As there are low anti-COVID-19 data, probiotic cultures could be recommended for respiratory activities and antiviral properties, becoming part of the alternatives to decrease the severity and burden of the disease. These authors highlighted that government funding has been used to test several drugs, but they also should fund probiotic trials.

The role of probiotics in asthma treatment was evaluated by Ahanchian et al. (2020) by using a systematic and meta-analysis review. The authors reported that the consumption of probiotics improved several parameters, such as the increase in the time between episodes of asthma, asthma control, and pulmonary function. However, the studies were highly heterogeneous, therefore, more studies are needed before considering probiotics as a strategy for treatment of the symptoms of asthma (wheeze) or prevention of respiratory infection or exacerbation in the patients.

Sundararaman et al. (2020) described the action of probiotic cultures in the modulation of the gut microbiota and gut homeostasis as well as in the production of interferons, which has been reported as a mechanism against virus. Additionally, these authors reviewed the function of probiotics on the lung-gut axis and immune system. The normal gastrointestinal microbiota of humans is populated with microorganisms of the genus *Lactobacillus* and *Bifidobacterium*. They are recognized as safe and commonly used in dairy products, such as yogurts (López-Moreno & Aguilera, 2020).

Pu et al. (2012) evaluated 205 participants aged >45 years for 12 weeks that were randomly distributed into two groups which received 300 mL/day of yogurt supplemented with a probiotic strain, *Lactobacillus paracasei* N1115 or not (control). Compared to the control group, the number of persons with an acute URTI (upper respiratory tract infections) and the number of URTI events significantly decreased in the intervention group. The risk of URTI in the intervention group was evaluated as 55% of that in the control group. The study reported that the probiotic yogurts (N1115 probiotic strain) may reduce the risk of acute infections in the upper tract in the elderly. The mainly underlined mechanism was the enhancement of the T-cell mediated natural immune defense.

Probiotics may balance the response to the defensive immune system of the host, stimulate the function of the mucosal barrier, and modulate the immune system. The intestinal bacteria may exert these beneficial effects by modulating the vitamin D axis, protecting the integrity of the mucosal barrier and suppressing the inflammation in the gut mucosa (Del Pinto et al., 2017). The interactions between gut and lung may impact on the role of probiotics in respiratory diseases, therefore, the influence on the immune system is considered a two-way process. The microbiota of the lung protects against infections in the respiratory tract, mainly by producing granulocyte-macrophage by the stimulation of Nod2 and IL-17. The study of Brown et al. (2017) verified that germ-free mice showed a higher morbidity in cases of lung infection, suggesting that gut microbiota has an important role. In the same way, the modulation of the severity of infections caused by *Mycobacterium tuberculosis* was correlated with gut microbiota (Namasivayam et al., 2018).

Some trace elements, such as zinc, can impact on the normal growth and development of the host, as it can alter the population of microorganisms in the intestine and has effect on the immune system, which was associated with the boost of the Th1 immune pathway. Zinc is absorbed in the gastrointestinal tract and there is secretion of endogenous zinc in the gut and its excretion in the feces. If this homeostasis is perturbed, the antiviral immunity is impacted. The deficiency of zinc can increase the risk of viral infections, as an imbalance of the Th2 and Th1 immunity functions can

be observed, resulting in a negative impact on the Th1 pathway. Leonardi et al. (2013) reported that LAB enriched with zinc could be an interesting source of zinc in food, as bioplexes or metalloproteins of zinc are recognized as the most suitable form for human absorption (Sundararaman et al., 2020).

Darbandia et al. (2021) elaborated a systematic review using randomized controlled trials (RCTs) to evaluate the effect of probiotic consumption on RTI. They reviewed 27 clinical trials with 9433 patients that had RTI and 10 clinical studies with patients with COVID-19. The results suggest the probiotic consumption may increase the cytokines levels in the plasma, the effect of the vaccine and the quality of life. Furthermore, it could reduce the duration and incidence of RTIs and the titer of viruses. The effects were attributed to the stimulation of the production of interferon; therefore, probiotic cultures may consider an adjunct therapy to prevent COVID-19. The authors recommend probiotics to complement the treatment of RTI diseases as an option to promote a faster recovery (Darbandia et al., 2021).

RTIs are originated by an imbalance in the microbiota of the gastrointestinal and respiratory tracts, which can affect the lung mucosa, alter the immune function, and increase the predisposition to a secondary infection by bacteria. The consumption of probiotic cultures may inhibit rotavirus, gastric coronavirus, and neuraminidase type 1 (H1N1) influenza virus, hemagglutinin type, and HIV. Furthermore, it can reduce the in vivo viral load (Anwar et al., 2020; Darbandia et al., 2021).

The safety of probiotics has been reported in several clinical studies. The consumption of probiotics and their derived factors can promote health effects and regulate the host homeostasis, including the immune system. Probiotics may protect from viral infection by modulating the host immune responses, keeping the gut homeostasis, and producing interferon, resulting in the suppression of the virus induced cytokine storm. Although a few randomized controlled trials showed that administration of probiotics could thwart pneumonia in COVID-19 patients, the impact on the mortality reduction needs to be proved. The administration of *Lactobacilli* and *Bifidobacteria* can overcome the gut dysbiosis induced by the SARS-CoV2 infection. The cytokine expression and immune stimulation are strain specific; therefore, they may vary depending on the consortia of the probiotic bacteria used. Therefore, a more targeted and novel approach to modulate the gut microbiota as a therapeutic approach of COVID-19 and the comorbidities will be needed. Research with probiotics and their possible mechanisms of action needs to be evaluated in clinical trials. These results suggest a demand of personalized medicine, and the clinical studies should evaluate the baseline microbiota of the individuals and their genetic pattern covering the responses after probiotic intake. In addition, biomarkers should be identified for the therapy's evaluation, including probiotics in hosts. Therefore, the immune stimulation provided by probiotics can prolong the resistance to infections by virus and reduce the risk of diseases in humans (Sundararaman et al., 2020).

16.4 Probiotics and gut-brain axis

Psychobiotic is any exogenous factor that is mediated by bacteria and acts on the central nervous system. Thus, it can include both probiotic and prebiotic, synbiotic and postbiotic agents. These microorganisms and substances may be obtained by the ingestion of functional foods, dietary supplements and a healthy diet (Long-Smith et al., 2020). The bacteria most used as psychobiotics are *Lactobacillus* and *Bifidobacterium*. In general, effects of psychobiotics are associated with: (1) psychological effects on cognitive and emotional processes through the direct action of some metabolites produced by the probiotic microorganisms; (2) systemic effects on stress responses, which involve an important modulation of the hypothalamic-pituitary-adrenal axis through the regulation of proinflammatory cytokine levels and normalization of glucocorticoid levels; and (3) neurochemical effects characterized by changes in the release rate of relevant neurotransmitters, such as gamma-aminobutyric acid (GABA), serotonin (5-HT), and glutamate (Cryan et al., 2019). Due to these actions, psychobiotics can be considered a new strategy for treating psychiatric and neurodegenerative diseases (Melo et al., 2020).

Depression is characterized by sadness, as well as reduced interest and pleasure in various activities. It occurs naturally in humans and is associated with situations of loss, defeat, and disappointment, among others. However, according to DSM-V (5th edition of the Manual of Diagnosis and Statistics of Mental Diseases), it becomes a psychopathological disorder, when this emotional state is exacerbated and lasts for more than 2 weeks, being accompanied by one of the following psychophysiological signs: sleep and appetite disorders, cognitive deficits, reduced self-esteem, pessimistic thoughts, psychomotor retardation, and suicidal ideas. This morbid condition, capable of compromising the individual's well-being, personal relationships, and job performance, is known as major depression disorder.

Depression affects 322 million people in the world, and the number has increased by 18.4% from 2005 to 15. The increase in the numbers follows the growth in the global population and is more prevalent in particular age groups. It is estimated that 4.4% of the global population had depression in 2015 (WHO, 2017). *B. bifidum, B. lactis, L. casei*, and *L. acidophilus* can regulate the microbial brain, and improve defects of memory, synaptic damage, and brain neurons in old

mice. Moreover, Chahwan et al., mentioned by Roobab et al. (2020), evaluated the effect of the consumption of probiotic foods on the function of brain, which was associated with depression. The authors reported that probiotic foods can improve depression, with a more prominent effect on men. However, the mechanisms of action were unable to assess this. Peng et al., mentioned by Roobab et al. (2020), evaluated the potential therapeutic effect of Lacidofil (95% *L. rhamnosus* and 5% *L. helveticus*) on children by analyzing the harmful effects of stress in early life, mainly anxiety symptoms and fear. The authors reported that the amygdala basolateral nucleus is a crucial node in the brain-gut axis and associated with the role of probiotics as therapeutic agents. It is important to understand the preferences, use, and attitudes concerning probiotics among children and caregivers; this is aimed for open discussions with pediatricians about the therapies involving probiotics, such as the probiotic efficacy, possible hazards, and health benefits (Roobab et al., 2020).

There are also many studies on humans that demonstrate differences in the fecal microbiota in patients with depression disorder when compared to healthy patients. In one of them, there was a reduction in bacteria of the genus *Bifidobacterium* and *Lactobacillus* in 43 patients with depression. In this context, some treatments with probiotics and psychobiotics have shown satisfactory results in improving depressive symptoms in both preclinical and clinical studies (Melo et al., 2020). Huang et al. (2016) published the first metaanalysis and systematic review aimed to determine the impact of probiotic consumption on depression. In this, one study worked with individuals with major depression, and the other four studies worked with nondepressed individuals. There was a high heterogeneity in the studies, which varied in dose, probiotic strain, duration, age of the individuals, therapies received, and depressive state. The authors reported that probiotics may decrease the risk of depression in nondepressed individuals. Furthermore, the impact was evident in individuals younger than 60 years, with reduction in the depression rating scales. There was no significant effect for individuals older than 65 years, suggesting that the effect was age dependent. However, it is important to mention that only one study worked with individuals older than 65 years, precluding a strong conclusion. The depression rating scales could also be reduced when probiotics were administered to healthy or depressive individuals, however, improvements in mood were only observed in individuals with several symptoms of depression at the baseline.

Chao et al. (2020) evaluated the effect of probiotics on depressive symptomatology or anxious in patients with an anxiety or depressive disorder diagnosis or under stress conditions by using a metaanalysis. The authors evaluated 10 clinical trials involving 685 individuals, which were assessed for the risk of bias (low or moderate). The results suggest that probiotics could decrease the depression scale in individuals with depression and anxiety, or in healthy individuals submitted to stress. However, no changes on the anxiety scores in individuals were observed. Furthermore, probiotics could reduce the depressive symptoms in depressive individuals, but had no effect in anxiety scores or performance under stress. The authors suggest that probiotics may be used as adjunct therapies for deep mental illness. However, they stated that clinical studies with a higher number of individuals are needed to prove the benefits of the novel treatment and propose the mechanisms of action (Chao et al., 2020).

A metaanalysis and systematic review considering double-blind, placebo-controlled, and randomized trials was performed to evaluate the impact of the ingestion of probiotics on symptoms of depression in humans. In the study of Goh et al. (2019), 19 studies (1901 individuals) were analyzed. The authors reported that probiotic consumption improved the depressive symptoms and can be considered a suitable approach to decrease the severity of depression. Furthermore, probiotics showed antidepressant impact in individuals with major depressive disorder, but no effect was observed for other clinical conditions or in the population in a general view. The authors also recommend the utilization of multiple probiotic strains to potentialize the effects in substitution to single probiotic strains. However, the results were considered preliminary, because the number of individuals with major depressive disorder was small and the probiotic species evaluated were diverse. They recommend that new clinical studies with a higher number of individuals and evaluating combinations of probiotic strains should be conducted (Goh et al., 2019).

Liu et al. (2019) performed a metaanalysis with 34 controlled clinical trials to evaluate the impact of the ingestion of probiotics and prebiotics on anxiety and depression. There was no effect of prebiotic on anxiety or depression, while probiotic consumption exerted a slightly pooled and significant effect. However, the authors reported that the evidence of probiotic and prebiotic effects on the treatment of internalizing disorders is modest. Furthermore, they state that although the results are qualified, there are scarce trials with psychiatric individuals and a prevalence of nonclinical individuals, which reduced the effects. In general, the most important results were observed for probiotic consumption and major depression, which was reported in four trials. The authors concluded that more clinical trials are needed to evaluate the efficacy of probiotics and prebiotics in anxiety and depression, and this is an extremely important are, mainly because there is a need of development of novel psychopharmacological agents for these diseases.

Obesity is a metabolic dysfunction and also a risk factor for psychological disorders. Agusti et al. (2018) evaluated whether *Bifidobacterium pseudocatenulatum* CECT 7765 could reverse the neurobehavioral consequences of obesity in a high fat diet-fed (HFD) mouse model via regulation of the gut-brain axis. They found that *B. pseudocatenulatum*

CECT 7765 has been considered a potential probiotic to alleviate depressive behavior (related to obesity) and social stress in animals. The mechanism of action was associated with the regulation of immune and endocrine mediators of the gut-brain axis. Additionally, this probiotic strain restored the leptin signaling and increased the weight loss.

16.5 Food allergy

Food allergy has received attention in recent years as it became a public health concern. According to Sicherer and Sampson (2014), cases of food allergy can represent more than 2%, but less than 10%, of the population and rates tend to be higher in children, Furthermore, it is one of the most stressors for families and results in impairment in the life quality of children. Finally, there is a substantial economic burden on the families, which is associated with the cost of health care (approximately $4184 per child with a food allergy per year), expenses with visits to doctors, medical treatment in case of emergencies and hospitalization, and decreases in productivity (Willits et al., 2018). It has been estimated that 5.4% of children younger than 18 years had some type of food allergy in the United States in 2014. Furthermore, 33% of children in Sweden and Denmark were affected by at least one case of allergy when aged 5 years old. Allergies show a substantial economic and health burden, and can decrease the life quality; therefore, the prevention of these diseases are of substantial importance (Schmidt et al., 2019).

Probiotics may be used in the treatment of food allergies in children and in adults. The evidence based on the medical literature show that probiotics, mainly *L. rhamnosus*, may decrease the symptoms of allergy to milk in children. Ma et al., mentioned by Roobab et al. (2020), applied probiotic mixtures and evaluated their effect in the reduction of food allergies and on the homeostasis of the intestinal immune system. Probiotics (*Bifidobacteria* and *Lactobacillus)* were considered a suitable strategy for treating food allergies. The authors reported that few studies have evaluated the effects of probiotics on food allergy, while many of them studied antibiotics. They stated that attention should be paid to the prescription of probiotics and antibiotics evaluating if the probiotic strains are not susceptible to infection. It is important to mention that many probiotic cultures, such as *Saccharomyces boulardii*, are resistant to the most common antibiotics. *L. plantarum*, *L. casei* Shirota, *L. rhamnosus* and *L. acidophilus* showed antipathogenic properties, which were dependent on the components of the matrix. A probiotic whey pearl millet-barley beverage, which was processed using an underutilized resource, prevented the growth of *Shigella* in animal models (mouse). Furthermore, *L. rhamnosus, S. boulardii*, and the combination of *L. rhamnosus, L. casei*, and *L. acidophilus* could reduce the infection by *C. difficile* (Roobab et al., 2020).

16.6 Probiotic health benefits on farm animals

The revenue from probiotics marked for animal feed has been estimated in United States at ~$2.6 billion in 2019, and an increase at a rate of ~7% is projected, reaching US$4.3 billion in 2027 (Transparency Market Research, 2019). Animals have microorganisms in the gut, which is denominated microbiome. With the reduction in time of analysis and costs of DNA sequencing and improvements in the data analysis pipelines, several investigations about the microbiome and its impact in health and disease were carried out. In these studies, metabolism, nutrition, immune function, development, and behavior were studied. The microbiome is used as a biomarker for health and disease conditions and could be correlated with the treatment of diseases. Studies involving the utilization of probiotic cultures by animals are in a higher number compared to human studies, mainly because host genetic diversity and diet can be more standardized, and animals are easier to manipulate experimentally. Furthermore, the regulations are generally less burdensome with animals compared to human research. Therefore, targeting the microbiome health in pets, livestock, and wildlife can have societal and economic impacts. The microbiome and its impact in the promotion of a healthier and more productive livestock has been promoted mainly because of the increase in the global population, with a consequent increase in the consumption of animal products (human population is estimated to be 10 billion in 2050). Furthermore, there is pressure to decrease the use of antibiotic in foods. Antibiotics have been extensively used as modulators of the microbiome, and they have been used for decades in aquaculture and livestock, increasing the safety concerns related to antibiotic resistance. This is commonly related to the fact that anaerobic gut commensals have the vanB locus, therefore, presenting vancomycin resistance. Furthermore, antibiotics are commonly used to increase feed efficiency and growth and prevent infections. Probiotics can have similar results in pigs, aquaculture, cattle, and chickens, being considered a suitable alternative to antibiotics (Jin Song et al., 2019).

The review of Al-Shawi et al. (2020) evaluated the effect of probiotic ingestion on the growth and development of animals, as well as immune response and productivity. Probiotic consumption resulted in improved feed efficiency and growth, lower mortality, and improved quality in the products. The mechanisms of action are not well understood, but

the modification of the microbiota is considered the main mechanism. However, the positive impact of probiotics on animal performance and the quality of meat products is variable because it depends on the probiotic strain, product stability during storage, form of administration, dosage, frequency of consumption, health status of the animals, type of diet, and age of the animal. The authors report that future studies are needed to find probiotic strains more effective for the desired use and determine the dosage, time of administration, method of delivery, and mechanisms of action.

Liepa and Viduža (2018) evaluated the impact of administration of *L. fermentum* additive on the metabolic diseases, ketosis I and SARA, in the early lactation period of dairy cows ($n = 240$). The groups presented healthy cows with conventional concentration of glucose and β-hydroxybutyrate. There was a control group and another group received the probiotic. *L. fermentum* improved the aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) activity and glucose in blood.

According to Uyeno et al. (2015) the use of probiotics in the feeding of adult ruminants improved the fiber digestion in the rumen. They also provided positive effects in different digestive system steps like cellulolysis and synthesis of microbial proteins. In the feeding of dairy cows the use of strains of yeast (usually *S. cerevisiae*) are used frequently, but for adult ruminants the genus *Enterococcus* and *Lactobacillus* are also applied. The most persistent effects after starting the use of yeast into the diet are enhanced productivity in lactating and growing animals. The mechanisms of action of these microorganisms has not yet been clarified, but is commonly considered to implicate modulation in rumen fermentation rates and patterns (Uyeno et al., 2015).

16.7 Health care costs and probiotics

RTIs caused by virus have an important burden on health care resources and society (Lenoir-Wijnkoop et al., 2019), as they affect individuals of all ages, resulting in absenteeism from school, daycare, and work (Lehtoranta et al., 2020). Lenoir-Wijnkoop et al. (2019) assessed the impact of probiotics on the economic (primary care setting) associated with RTI events. They evaluated the results provided by two metaanalysis and published by YHEC and Cochrane. These studies demonstrated the efficacy of probiotics in the reduction of duration and incidence of RTIs, quantitative of antibiotic courses, and decreases in the days absent from work. The authors used a state-transition microsimulation model to reproduce a representative population of the United States for gender and age. The incidence of RTI was related to illness similar to influenza, which were reported by CDC FluView. The data of vaccination, factors that impact RTI outcomes, resource use, and loss of productivity were retrieved from United States databases. The results demonstrated that probiotic utilization may reduce the costs associated with health care, reduce the number of episodes of RTI, decrease the number of consultations by patients, decrease the medical prescriptions, and decrease the productivity loss. In general, the utilization of probiotics by US population (2017−18) would save US$4.6 million for health care in the YHEC study, and US$373 million in the Cochrane study, and could also invert 19 million and 54.5 million sick days, respectively. The utilization of antibiotics could decrease to 1.39−2.16 million courses, and the absence of work would decrease to 3.58−4.5 million days. If loss of productivity were included, the total saving would be US$784 million and $US1.4 billion for the YHEC and Cochrane studies, respectively. The results also demonstrated that the benefits in at-risk groups was important, which suggests that target interventions could be carried out. Therefore, the authors concluded that probiotics may impact positively on the economic burden (for payer and society) and health care of RTI.

A common cause of morbidity is bacterial infection after a gastrointestinal surgery. Yang et al. (2017) aimed to evaluate the effect of probiotics and synbiotics (perioperative use) on postoperative infections. A total of 28 studies including 2511 individuals were included in the systematic review and the variables were the rate of postoperative infection, the length of stay at hospital or intensive care unit, the duration of the therapy with antibiotics, and the mortality. The individuals who received probiotics showed a lower incidence of complications caused by infections, mainly associated with urinary, respiratory, and wound infections. Furthermore, the duration of the antibiotic therapy and hospital stay were shortened. No significant differences in the mortality were reported. The authors concluded that probiotics/synbiotics may reduce the risk of infections after gastrointestinal surgeries. However, the results should be interpreted with caution, mainly because of the risk of bias in the evaluated studies or publication bias (Yang et al., 2017).

Probiotics may reduce the risk of infections associated with health care, such as pneumonia from ventilator, diarrhea associated with *C. difficile*, among others. Lau et al. (2020) performed a systematic review (seven studies) to evaluate the economic effect of the use of probiotics in hospitalized adult individuals, being one RCT and six model-based health economic studies. Most of the studies (86%) reported effectiveness of probiotics on the reduction of costs. The authors suggest that future studies should include costs related to probiotics and clinical outcomes, so that clinical guidelines, bedside practice, and health care policy could be informed. It is important to mention that the control and

prevention of infections associated with health care depend on several factors, such as the utilization of transmission and standard-based precautions, adequate hygiene of the environment, suitable diagnostics, and use of antibiotics with prudence. These infections are a global worry, as they impair the patient outcome, prolong the hospital stay, and increase the costs of health care (Caselli et al., 2019; Facciolà et al., 2019).

The utilization of detergents or disinfectants in conventional sanitation techniques cannot prevent the recontamination and, in some cases, may select pathogens and increase the antimicrobial resistance. It was proposed that the utilization of detergents with spores of probiotic *Bacillus* could reduce the number of surface pathogens, presenting higher performance compared to conventional sanitizers. Furthermore, it did not induce the selection of resistant microorganisms, which was evaluated by molecular analyses of the resistome of the entire microbiota. The utilization of the detergent was also associated with a 52% reduction in health care-associated infection incidence in hospitalized individuals. The authors reported some limitations of the study, such as data analyzes were performed without comparing the patients (groups could not be identical), only patients with complete data of drug therapy were included (could have underestimation of the consumption of drug and costs), and only drug costs were evaluated (other parameters should be included, such as labor costs, apparatus used, bed day costs) (Caselli et al., 2019).

16.8 Challenges for the future and final considerations

Further research is required to evaluate the effect of probiotics on the human microbiota and how they modify the levels of biomarkers and cause improvements, mainly by using high-throughput methodologies. Future studies should elucidate how the administration of probiotics could integrate the impact on diseases that are multifactorial, considering the pathophysiological status, predispositions for developing metabolic diseases, history of dietary xenobiotic obesogens exposure, clinical and genetic factors, and precedent evaluations of the specific patient features. Furthermore, it is important to motivate the publishing of the clinical trials in open access literature, increasing the availability of data and enhancing the comparisons. Finally, new species of next-generation probiotics should be evaluated as preventive and therapeutic alternatives (López-Moreno et al., 2020).

The safety of probiotics is well established in several clinical trials, as well as their ability to provide health benefits and regulate the host homeostasis, including the immune system. Furthermore, probiotic cultures may have antidepressant and anxiolytic properties, but additional clinical studies with psychiatric individuals are needed to fully understand the therapeutic potential.

To identify new probiotics for animal use, there is a need of high-throughput culturing of a broad phylogenetic microorganism from several animal hosts. This is important because of all microbial genera, only 37 can be found in probiotic products, and they focus on bacteria rather than fungus. In studies concerning the possible health effects, it is important to determine the mechanisms of action of probiotics, aiming to provide therapeutic strategies that could be generalized. In this way, organoids may be an exciting avenue to study host- and species-specific interactions between microbes and tissues, including many animal species that are impractical to be kept and bred in the laboratory.

All the research efforts will be important to clarify even more the essential function of probiotics to human and animal health. The probiotics present in dairy products, other food products and supplements can improve human health savings costs in the health care system and improve the economy. In the same way, probiotics in feed can save costs in the breeding practice and contribute to produce more safety animal products.

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Chapter 17

Recent advancements in the production of probiotic fermented beverages

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17.1 Introduction

Increasing awareness to maintain and improve health and wellness leads people to eat healthy. These healthier foods, viz. functional foods, have physiological effects on the body and provide health benefits besides providing nutritional requirements because of the presence of beneficiary microorganisms in them (Argan et al., 2015; Bhadoria & Mahapatra, 2011; Guneser et al., 2019). Functional foods are also known as nutraceuticals. The functional food category that is increasing in demand is dairy-based probiotic fermented beverages.

The word *probiotic* was coined from the Greek meaning "for life" (Fuller, 1989). The product comprises live microorganisms and is capable of increasing good bacteria in the intestine upon ingestion, which is termed as "probiotics" (Bhadoria & Mahapatra, 2011). For the first time, Lilly and Stillwell in 1965 used the word probiotic. They coined the term for the substances produced by one microorganism which encourage the growth of others (Lilly & Stillwell, 1965). Modern definitions keep more emphasis on the preventive or therapeutic actions of probiotics (Soccol et al., 2014). Recently, the FAO/WHO (Food and Agriculture Organization/World Health Organization, 2006) recognized by the International Scientific Association for Probiotics and Prebiotics (Reid et al., 2003), defined probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001, 2002; Hill et al., 2014). Thus, probiotics are single or mixed cultures of live microorganisms that upon consumption in adequate amounts confer health benefits to host animals. Hence, the maintenance and/or improvement of the numbers and properties of indigenous microorganisms of the intestine played a major role in improving the host health (Bhadoria & Mahapatra, 2011; Fuller, 1989; Tabbers & Benninga, 2007).

Dairy beverages are manufactured using milk or milk derivatives by fermentation using yogurt culture. In this, the milk or its derivatives are used alone or with other added ingredients, but the minimum concentration of dairy base should be 51% (v/v) (Castro et al., 2013). Milk is considered an important part of the human diet right from birth; it provides all the essential nutrients required for the growth and maintenance of the human body. Thus, milk with an abundance of minerals (i.e., P, Mg, Zn, K, Ca), essential fatty acids, vitamins, isoflavones, conjugated linoleic acid, and phytosterols have been widely used in the development of functional foods (Aureli et al., 2011; Hati et al., 2019; Heenan et al., 2002; Sartor, 2004). Milk-based beverages occupy 43% of the functional food market (Argan et al., 2015; Kelly et al., 2009; Mellema & Bot, 2009; Özer & Kirmaci, 2010). The increasing demand for milk-based beverages provides us with the opportunity to develop new products incorporating desirable nutrients and bioactive compounds (Corbo et al., 2014; Sloan & Hutt, 2012; Sorenson & Bogue, 2009). Dairy products can be fermented by a diverse microbiota (Macori & Cotter, 2018).

Fermented foods are made by controlling the growth of complex microbiota (naturally occurring indigenous or selected) and the metabolic activity of microorganisms, that is, enzymatic conversion of complex food components into simpler small components (Marco et al., 2017). The microorganisms can be a few of the broken constituents that might possess bioactive properties. Thus, fermentation results in enhanced nutritional and functional properties such as easy availability of nutrients, more safety, increased self-life (food preservation due to the organic acid production), and also imparts pleasant sensory (confers enhanced organoleptic) properties, that is, texture, aroma, and flavor and can also add

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nutritional and health-promoting attributes to the foods (Hati et al., 2019). Fermented food is considered the healthiest food and microorganisms present in fermented food reside in the intestine upon consumption (Ross et al., 2005). Fermented milk is recognized as a competent probiotic carrier (Hati et al., 2019).

17.2 Dairy-based probiotic fermented milk beverages

17.2.1 Merits of dairy-based beverages as probiotic carriers

Amongst all probiotic beverages, fermented dairy drinks were the very first to be commercialized and are still utilized in huge amounts today (Hati et al., 2019).

Dairy-based beverages are considered very active functional foods because:

It is proved to be a good carrier of probiotic organisms. It provides nutrients and bioactive components for the growth of organisms.
It fulfills numerous consumer demands.
It is easy to distribute.
It can easily be stored in refrigerated storage conditions (Kausar et al., 2012).
It is well evidenced that the type of carrier food affects the viability of probiotics during processing, storage, viability, and functionality in GI transit under the adverse conditions of acid, bile, and enzymes, ability to adhere to gut epithelial lining and immune modulation (Marco & Tachon, 2013; Ranadheera et al., 2012, 2014). Dairy-based carrier foods prevent probiotics from harsh GI conditions more efficiently as compared to other nondairy carrier foods because of the buffering action of milk and milk fats and protection of probiotics from the direct exposure to harsh conditions of the GI (Ranadheera et al., 2010).

17.2.2 Classification of milk-based beverages

Milk-based beverages are categorized into two as follows (Jelen, 2009):

1. Unfermented milk and milk derivatives: such as flavored milk, fortified milk (added with sterols, fish oil, fibers, etc.).
2. Fermented milk products: *kefir*, *koumiss*, *yakult*, buttermilk, and fermented milk with probiotics. This group of products is mass-produced in a well-controlled complex fermentation process.

More than 400 commercial milk-based probiotic fermented products are available globally under different brand names. Fermented milk products are classified based on major physicochemical properties which differ based on the milk type, probiotic microbes involved and the nature of starter cultures (the strain used for fermentation) in the product, and the majority of sensory metabolites as shown in Robinson and Tamime (1996) and Shiby and Mishra (2013).

A series of fermented dairy-based beverages like *lassi*, acidophilus milk, *bifidus* milk, acido-whey, *kefir*, and various whey-based beverage and semisolid products are widely consumed by the public and a high demand also reported in the market (Kent & Doherty, 2014; Ranadheera et al., 2017; Soukoulis et al., 2014).

Fermented milk is subdivided into three types as shown in Table 17.1 based on fermentation temperature ranges used for its production: thermophilic sour-milks (42°C−45°C), mesophilic sour-milks (20°C−30°C), and acid and alcoholic milk (15°C−25°C).

This can further be diversified by

using milk from different species of animals and breeds,
adding sugar, fruits, condiments, and grains, and
applying different preservation technologies (as drying, concentration, or freezing) (Valls et al., 2013) (Fig. 17.1).

17.3 Challenges for production of probiotic fermented dairy beverages

Numerous challenges are in production as well as in the development of new fermented probiotic dairy beverages with new functionalities, that is, health benefits, as follows:

Isolation and screening of technologically suitable probiotic strain which should (Fenster et al., 2019; Ross et al., 2005; Saavedra et al., 1994; Talwalkar & Kailasapathy, 2004; Ventura & Perozzi, 2011):

- be safe;

TABLE 17.1 Traditional fermented dairy products along with the associated organisms.

Type of product	Name of product	Source of milk	Type of starter	Some associated microorganisms involved in fermentation	Health benefits
Cheese	Cheese varieties	Milk types (buffalo, cow, goat, mare, sheep)	Traditional and commercial	Various including *Lc. lactis*, *Lactococcus* spp., *Lactobacillus* spp., and *Streptococcus* spp	Intestinal probiosis; production of bioactive peptides
Sour milk Thermophilic	Ayran	Cow	Traditional and commercial	*Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) and *Streptococcus salivarius* subsp. *thermophiles* (*Str. thermophilus*)	Rich in nutrition and desirable organoleptic features
	Matzoon	Cow	Back-slopping (culture from the previous production)	*Lb. bulgaricus* and *Str. thermophilus*	Stimulate the function of the pancreas and liver.
Fermented milk Mesophilic sour milk	Cultured buttermilk	Boiled cow or goat milk	Traditional and commercial	*Lc. lactis* subsp. *Cremoris* and *Lc. lactis* subsp. *lactis biovar. diacetylactis* (*Lc. Diacetylactis*)	Lower the cholesterol; Improve the oral health
	Nunu		Traditional and commercial	*Lb. fermentum*, *Lb. plantarum*, *Lb. helveticus*, and *Leuconostoc mesenteroides* (*Leuc. mesenteroides*)	Improving the nutritional status of consumers
	Skyr	Skimmed sheep and cow milk	Traditional	*Str. thermophilus* and *Lb. bulgaricus*	Antioxidant, hypotensive, and immunological effects
	Villi	Cow	Back-slopping (culture from the previous production)	*Lc. lactis* subsp. *cremoris*, *Lc. diacetylactis*, *Leuc. mesenteroides* subsp. *cremoris Geotrichum candidum*, *Kluveromyce smarxianus*, and *Pichia fermentans*	Antiinflammation, antitumor, and immunomodulation activities.
	Ymer	Slimmed cow milk	Traditional	*Lc. lactis*	Improve the bone health; rich with calcium, iron, and other essential nutrients
	Gioddu	Cow, goat, sheep, and mixed milk	Back-slopping (culture from the previous production, called madrighe)	*Str. thermophilus*, *Lb. bulgaricus*, *Lc. Case i*subsp. *casei*, and *Leuc. mesenteroides* subsp. *mesenteroides*	Rich with antimicrobial compounds and bioactive compounds
Fermented milk acid and alcoholic milk	Kefir	Any kind of milk (buffalo, camel, cow, goat, sheep) and mixed milk	Back-slopping (culture from the previous production, "grains")	*Lb. acidophilus*, *Bifidobacterium bifidum Str. thermophilus*, *Lb. bulgaricus*, *Lb. helveticus*, *Lb. kefiranofaciens*, *Lc. lactis*, *Leuconostoc* spp., *K. marxianus*, *Micrococci*, *K. lactis*, *Saccharomyces kefir*, *S. cerevisiae*, and *Torula kefir*	Antistress, immunomodulation; enhance the gut microbial growth
Yogurt	Traditional yogurt	Cow	Traditional and commercial	*Str. thermophiles*, *Lb. bulgaricus Lb. helveticus*, *Lb. fermentum* and *Lb. paracasei*	Antiobesity, reduced body weight; stimulate the growth of gut microbiota

(*Continued*)

TABLE 17.1 (Continued)

Type of product	Name of product	Source of milk	Type of starter	Some associated microorganisms involved in fermentation	Health benefits
Acidophilic milk	Acidophilus milk	Cow	Traditional	*Lb. acidophilus*	Reduce cholesterol; antiobesity; prevent vaginal infections
	Koumiss	Mare	Back-slopping (culture from the previous production)	*Enterococcus faecalis*, and *E. faecium*	Treat weight loss and anemia; reduction in colon cancer
	Shubat or Chal	Camel	Traditional	*Lb. paracasei*, *Lb. helveticus* and *Str. thermophilus*	Antiobesity
	Gariss	Camel	Back-slopping (culture from the previous production)	*K. marxianus*, *Candida kefyr*, *Pichia kudriavzevii*, *Str. infantarius* subsp. *infantarius*, and *Lb. fermentum*	Antibacterial potential
Fermented milk	Susac	Camel	Traditional	Lactic acid bacteria	Antimicrobial, antioxidant, and anticancer activities

Lc., Lactococcus; Str., Streptococcus; Lb., Lactobacillus; Leuc., Leuconostoc; K., Kluveromyces; E., Enterococcus.
Source: Adapted from Aryana, K. J. & Olson, D. W. (2017). A 100-year review: Yoghurt and other cultured dairy products. *Journal of Dairy Science*, 100, 9987–10083; Macori, G. & Cotter, P. D. (2018). Novel insights into the microbiology of fermented dairy foods. *Current Opinion in Biotechnology*, *49*, 172–178; Settani, L. & Moschetti, G. (2010). Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiology*, *27*, 691–697 (Aryana & Olson, 2017; Settani & Moschetti, 2010).

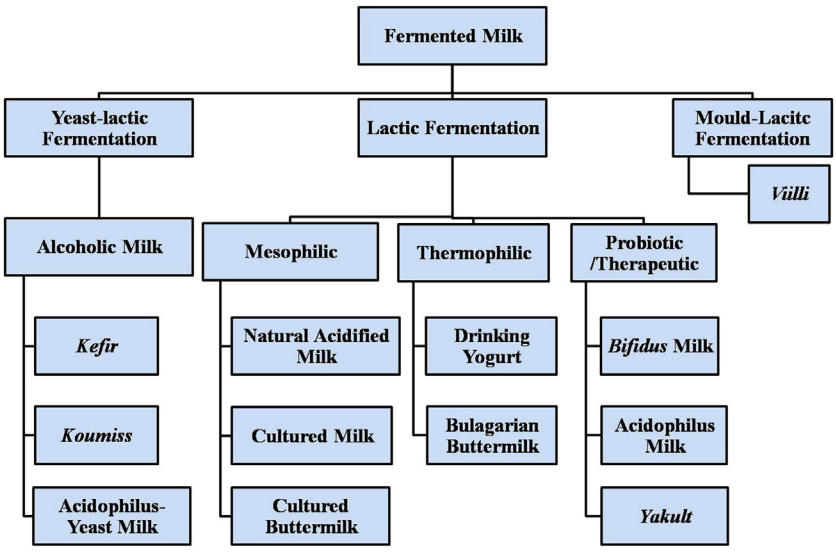

FIGURE 17.1 Classification of probiotic fermented dairy beverages based on the type of fermentation/major physicochemical properties (Ranadheera et al., 2017; Tamime et al., 2007).

- remain viable and maintain functionality during production and processing operations, storage, and gastrointestinal transit;
- grow and survive and maintain functionality in fermented dairy beverages at large scale industrial product; and
- have good sensory and organoleptic properties.

Though plenty of clinical evidence supports the positive health-promoting effects of probiotics on humans, the technological suitability of these strains limits their exploitation (Ross et al., 2005). Bacteria with good technological properties can be produced and added to food products with exceptional functional health properties. Definition of probiotics addresses three prime criteria that should be fulfilled: dose, viability, and health effects. Out of which dose and viability are associated with the production technologies (Saarela, 2009).

17.3.1 Isolation and screening of strain which should be technologically suitable

As mentioned previously, considerations for screening microorganisms to be valuable probiotic strains are:

1. The strain should be viable and metabolically active within the GI tract, that is, it should survive transit through the stomach; acidic conditions of the stomach and the presence of bile salt.
2. The strain should be viable and maintain its desirable characteristics throughout the manufacturing and in the final product (Godward et al., 2000; Talwalkar & Kailasapathy, 2004).

Careful screening allows the selection of technologically suitable strains that have the best manufacturing and food technology characteristics. Though, sometimes the most robust organism remains imperfect in the food applications for which it is selected. When selecting an organism, the question arises of whether to consider the microorganism as probiotic or not. When passing through the stomach, viability and metabolic activity of organisms are maintained and remain very much active in the intestine, the organism can be claimed as a probiotic. However, certain basic criteria are used for designating a strain as a probiotic, as mentioned below (Havenaar & Huis, 1992; Hyun & Shin, 1998; Iacono et al., 2011):

1. It should be of human origin.
2. It should survive during gastrointestinal transit in the human intestinal tract.
3. It should have acid and bile stability.
4. It should adhere to the human intestinal cells.
5. It should possess the capacity to produce antimicrobial substances.
6. It should show antagonism against pathogens.
7. It should possess immune modulator activity.
8. It should be safe for the host in food during clinical use.
9. It should be resistant to antibiotics.
10. It should tolerate food components and should be stable in a food matrix.
11. It should have clinically validated health benefits.

However, all the probiotics used nowadays were not selected using the above-mentioned criteria (Nole et al., 2014). For the past two decades, fermented dairy products with added human intestine-originated probiotic bacteria are preferred (Hati et al., 2019). The most common genera and species used are LAB such as *Lb. acidophilus*, and *Bifidobacteria* (Daly & Davis, 1998). They are reviewed as Generally Recognized as Safe (GRAS) (Butel, 2014; Millette et al., 2013; Rivera-Espinoza & Gallardo-Navarro, 2010). They are the natural leading inhabitants of the human gastrointestinal tract (the presence of *Lactobacillus* and *Bifidobacterium* in the small and large intestines respectively) (Rivera-Espinoza & Gallardo-Navarro, 2010). However, sometimes they show low tolerance to stress, which leads to a reduction in their viability in probiotic products (Ozen et al., 2012).

The probiotic effect and acid and bile tolerance of microorganisms and endurance against different damaging aspects of product manufacturing are species and strain-dependent (Tamime et al., 2005); therefore, it must preferably be assessed using different acid-bile tolerance assays, identified phenotypically, genotypically and characterized (Ross et al., 2005). The choice of suitable strains by their acid and bile tolerance might help in improving the viability of probiotic organisms (Takahashi et al., 2004).

Many strains of *Lb. acidophilus* and *Bifidobacterium* spp. could not remain viable under the harsh conditions of the GIT. Some strains might not be suitable to use as dietary aides in fermented milk. Yet some strains are reported to tolerate acidic conditions and moderate bile concentrations (Clark & Martin, 1994; Clark et al., 1993). For example, upon exposure at pH 2 for 45 minutes, all cells of *Lb. acidophilus* died, whereas, upon exposure at pH 4 for 4 hours, there was no significant reduction in the numbers of cells (Hood & Zoitola, 1988). Similar observations are found for the viability of bacteria at pH 1−7 especially *Lb. rhamnosus* GG (Goldin et al., 1992). *Bifidobacterium longum* and *B. pseudolongum* showed the best tolerance to acid and bile salts (Lankaputhra, 1995) compared to *B. infantis*, *B. adolsecentis*, and *B. bifidum*. Furthermore, *B. longum* can easily be grown in milk, and *B. animalis* subsp. *animalis* have adequate persistence properties in fermented milk. But, *B. animalis* subsp. *animalis* is of nonhuman origin (Lankaputhra & Shah,

1996). Besides having low tolerance to acid and bile, *Bifidobacteria* are anaerobic. Hence, a high oxygen concentration in probiotic products may affect their growth and viability.

Lactobacilli are generally found to be more robust (more tolerant to acidic conditions) than *Bifidobacteria* (Mättö et al., 2006; Ross et al., 2005). The growth of *Bifidobacteria* remarkably deaccelerates below pH 5.0, which has been demonstrated using human gastric juice (Dunne et al., 1999). *Lactobacilli* are found to be well-tolerated to low pH and can grow easily in milk and other food substrates (Tripathi & Giri, 2014). The reported stability of *Lb. acidophilus* under acidic conditions is due to a high cytoplasmic buffering capacity (pH 3.72−7.74). This allows organisms to resist alteration in cytoplasmic pH. Thus, probiotic *Lactobacillus* species are technologically appropriate compared to *Bifidobacteria* (Lee & Salminen, 2009).

When *Lactobacilli* are isolated from the harsh environs of gastro intestinal tract (GIT), they are acid-tolerant or aciduric (McLauchlan et al., 1989). Similarly, *Bifidobacterium* isolated from human fecal showed both acid and bile tolerance (Chung et al., 1999). Thus, the stressed cultures demonstrated good survival under acid and bile atmosphere. With the same approach, acid and bile resistant *Lb. acidophilus* variants with distinct characteristics compared to parent strains have been isolated (Chou & Weimer, 1999). Also, acid-adapted *B. breve* was found to exhibit greater endurance features in contrast to nonadapted cells (Park et al., 1995) under acid, bile, hydrogen peroxide, and cold storage.

Microorganisms such as *Lactococcus*, *Bacillus*, *Propionibacterium*, *Enterococcus*, yeasts (i.e., *Saccharomyces cerevisiae* and *Saccharomyces boulardii*), and filamentous fungi (i.e., *Aspergillus oryzae*), having health-promoting effects in fermented food products including dairy (Barbosa et al., 2011; Cousin et al., 2012; Ozyurt & Ötles, 2014; Tripathi & Giri, 2014).

Furthermore, few scientists recommended supplementation of probiotics with multispecies. This might have a more specific function in the human alimentary tract (Saxelin et al., 2010).

17.3.2 Starter cultures

Production of fermented milk products requires considerations of several aspects as follows:

1. growth of several probiotic organisms are slow in nonsupplemented/augmented milk;
2. conventional probiotic manufacturing conditions such as temperature often inappropriate for the growth; and
3. certain metabolites produced by probiotic microorganisms may generate off-flavor which is not desirable, for example, *Bifidobacteria* produce acetic acid that leads to vinegar-like taste (Gomes & Malcata, 1999; Østlie et al., 2003; Saarela et al., 2000; Saxelin et al., 1999).

One can lower the production cost of probiotic fermented milk if the milk supports the growth of probiotic organisms and no off-flavor is developed.

The probiotic culture used in the fermented probiotic product must contribute to good sensory properties. The common strategy is to use probiotic organisms with other types of bacteria known as a starter culture, which can ferment a specific product. Studies have shown that using probiotics along with starter cultures, fermented dairy products with excellent sensory properties can be produced (Mattila-Sandholm et al., 2002). Thus, starter cultures that help in improving the flavor and texture of fermented food products are of great industrial significance (Ranadheera et al., 2017). When selecting probiotics, the criterion is an impact on human health and wellbeing, similarly, in choosing an appropriate starter culture, the acid-forming ability is an important criterion.

Suitable starters used are *Streptococcus thermophilus*, yogurt cultures, and mesophiles with different combinations of *Lactococcus* strains (Senok et al., 2005). *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* are used as starter cultures for the production of yogurt. In some fermentation processes to enhance the fermentation process, yogurt starter culture is added. *Lb. acidophilus*, *Bifidobacterium* spp. and *Lb. casei* are combined as dietary adjuncts (Leroy & De Vuyst, 2004; Minelli et al., 2004; Saito, 2004).

Fermented milk can be manufactured with only *Lb. acidophilus* or *Lb. acidophilus* and *Bifidobacterium* spp. (known as AB cultures), *Lb. acidophilus*, *Bifidobacterium* spp. and *Lb. casei* (known as ABC cultures) (Maiocchi, 2001), or *Lb. acidophilus*, *Bifidobacterium* spp. and *S. thermophilus* (known as ABT cultures) (Martín-Diana et al., 2003). The period of incubation and quality of the product, include flavoring fermented dairy products with the bacterial consortia AB, ABC, or some blends of ABT cultures (i.e., ABT-1 and ABT-2). Hence, the common practice is to use *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and probiotic microorganisms (AB or ABC cultures) for the production of yogurt (Tamime et al., 2005).

It is required to use compatible and suitable combinations of probiotic and starter cultures in circumstances when using them simultaneously during fermentation (Ouwehand et al., 2000; Saxelin et al., 1999). The main criteria for the selection of probiotics as a starter culture are health benefits to humans (Gardiner et al., 2002). However, as a manufacturer of starter culture, the following criteria must be measured:

1. the probiotic microorganisms should be able to grow in a fermentation medium to increase the cell counts;
2. the organism should survive the freezing and drying stages of preservation; and
3. the organism should tolerate stomach harsh conditions of acid and bile and survive the GI transition.

Probiotics are generally not appropriate starter cultures because of the different environments of GI tract and food (German et al., 1999; Oberman & Libudzisz, 1998). Besides, the slow growth rate might impart off-flavors (Tannock, 1999). Several trials have been performed using milk as a substrate for probiotics, so that they can be used as a starter culture by incorporating important energy sources including glucose, vitamins, minerals, and antioxidants (Dave & Shah, 1998; Gomes et al., 1998; Ishibashi & Shimamura, 1993; Kurmann, 1988). Though improvement in the performance of probiotics as starter cultures have not been observed enough. This can be achieved by using a starter culture along with a probiotic preparation (Alander & Mattila-Sandholm, 2000).

Due to less survival of starter cultures in the digestive tract, the word "probiotics" may not be suitable for yogurt starter cultures (*S. thermophilus* and *Lb. delbrueckii* spp. *bulgaricus*) (Senok et al., 2005). On the contrary, yogurt starter cultures have been reported to have some beneficial health-promoting effects, counting enhanced lactose consumption and improvement of the immune system (Guarner et al., 2005; Meydani & Ha, 2000).

The blending of a starter culture and probiotic strain for the production of fermented milk products is a crucial step. Trials are performed to confirm the desired characteristics in finished products. The key factors to consider in this regard include duration of fermentation, mildness, texture, tolerance to sugar concentration and postacidification profiles (Tamime et al., 2005). But the most considerable attribute for dairy-based fermented probiotic products is the stability of probiotic strain. Currently, the trend has been set for the probiotic product that it should have a longer shelf life of up to 52 days to have health benefits (Tamime et al., 2005). Therefore, the blend of probiotic/LAB is checked in laboratories for the stability of probiotic microflora for 28 days of shelf life at 8°C. The main aim of the experiment is the product should have a minimum count of 1×10^6CFU/g at the end of the storage period. If not, the mixture of organisms is developed again (Tamime et al., 2005).

Probiotics may also affect the flavor of fermented foods. For instance, *Bifidobacterium* spp. at higher concentrations produce acetic acid in long-run fermentations (Mahdi et al., 1990; Torre et al., 2003). Similarly, *Lb. acidophilus* produces acetaldehyde and lactic acid leads to the characteristic "bio" yogurt flavor. High proteolytic probiotic organisms might produce peptides, that confer a cheesy flavor to the fermented milk product (Rasic & Kurmann, 1983).

When both probiotics and starters are to be used simultaneously during fermentation, the most appropriate blend of starter and a specific probiotic bacterium has to be evaluated based on the effect of diverse starter cultures on the sensory properties and the endurance of the probiotics (Ishibashi & Shimamura, 1993; Samona et al., 1996; Saxelin et al., 1999). Starter cultures might be advantageous or disadvantageous in probiotic products. Starters may produce growth factors or reduce oxygen content and thus improve the growth and survival of probiotics (Dave & Shah, 1997a, 1997b; Kailasapathy & Rybka, 1997; Saarela et al., 2000; Saxelin et al., 1999; Vinderola et al., 2002). For example, during yogurt manufacturing, starter culture *Streptococcus thermophiles* are found to scavenge oxygen, therefore, creating a suitable environment for growth and improve the viability of anaerobic probiotic bacteria. *Streptococcus thermophiles* have proven to complete milk fermentation within 5−10 hours. Within this time, it was found to utilize most of the milk oxygen (Dave & Shah, 1997a).

Also, a change in starter combination may affect the viability of probiotics in the finished product due to antagonistic or symbiotic relationship (Ranadheera, 2011). Metabolites such as high lactic acid, hydrogen peroxide, and bacteriocin, produced by starter cultures, might have a negative influence on the growth decreases the viable count of probiotics (Katla et al., 2001; Mattila-Sandholm et al., 2002; Vinderola et al., 2002).

Certain fermentation products are produced at 20°C−30°C, but this is a suboptimal temperature for probiotic growth (probiotic strain which is originated from human GIT, has an optimum growth temperature of 37°C). Increasing temperature for enhancing the growth of probiotics is not suggested as it may produce off-flavors in the products (Mantere-Alhonen & Forsen, 1990). Hence, the blending of probiotic organisms with a thermophilic starter culture (e.g., a blend of *Lb. acidophilus* and/or *Bifidobacteria* and yogurt starter cultures) might provide good products (Gardini et al., 1999; Saarela et al., 2000; Saxelin et al., 1999).

17.3.3 Dose

The primary requirement for the development of probiotic food is the production of a strain in adequate quantity. At the time of consumption, the product must have an appropriate organism in sufficient numbers in viable condition to get the desired health benefits (Korbekandi et al., 2011; Tripathi & Giri, 2014). Though the acceptable number of

microorganisms is not specified in the definition, for successful delivery in foods, the minimum recommended dosage of viable probiotic cells per day is approximately 10^8-10^9 probiotic colony forming units (CFU)/mL/CFU/g. This concentration figure is associated with an intake of ~100 g of fermented food product containing 10^6-10^7 CFU/mL or /g per day (Hill et al., 2014; Ishibashi & Shimamura, 1993; Lee & Salminen, 1995; Lourens-Hattingh & Viljoen, 2001; Sreeja & Prajapati, 2013; Walsh et al., 2014). The acceptable quantity is supposed to be at least the dose that has been reported to offer desired health benefits. Nowhere it has been reported that a higher dose is detrimental (Morovic et al., 2017; Zhou et al., 2000). Even in certain cases, it has proved to be beneficial (Ouwehand et al., 2018). Furthermore, one should consume the probiotic food product regularly in appropriate amounts to bring the decided/desired "dose" of live bacteria in the gut. Here, losses of cell viability during GIT transit should also be considered.

The most critical challenge during manufacturing and incorporating probiotics in products is to develop food that should have sufficient doses of probiotic organisms at the end of shelf life and at the time of consumption. For successful delivery of probiotics in food, it is expected that the organisms survive and retain functionality in production-storage conditions, tolerate acidic conditions of the stomach, and should be resistant to enzymatic degradation and bile salt in the small intestine (Fenster et al., 2019; Stanton et al., 2003). In dietary supplements, during transportation and storage at ambient temperature and humidity, loss of viability is more in comparison to refrigerated storage. Also, the probiotic strains should be incorporated in products that let them survive in adequate quantities until the end of shelf life. To rectify all the above-mentioned challenges, it is recommended to add an excess of probiotic organisms to the product.

17.3.4 Viability

Maintenance of viability during food processing, storage, transportation, and GI transit is vital for the probiotic organisms to reach the actual site of action in the appropriate quantity. Factors influencing the survival and stability of probiotics in beverages are as follows (Saarela, 2009):

1. Probiotic strain: its characteristics and the form in which it has been added to beverages.
2. Probiotic food base (milk and milk derivatives): chemical composition(some might be nutrients and some antinutritive), pH, presence of organic acids, additives.
3. Organisms: starter culture or other probiotic organisms in a blend of organisms (inhibitory or growth enhancer).
4. Packaging with oxygen barrier or penetrating properties.
5. Storage time and temperature.

17.3.4.1 Viability during production-processing operations and storage

The probiotic manufacturing process should be such that it confirms high yield and stability. Also, the process should not produce specific allergens, which means certain media ingredients should be precluded (Fenster et al., 2019). To confirm consistent high performance and reproducibility, quality control is essential during the entire production process, which requires extensive skill and experience. Probiotic viability losses occur if the microbes encounter stressful conditions. Maintenance of optimal growth conditions for the organism in batch fermenters is difficult as the concentration of inhibitor substance changes constantly (Rallu et al., 1996). Also, different processing steps of probiotic production (such as harvesting, freezing, drying, and other manipulations) induce stress to microbes. Also, the matrix to which the strain is added—pH, gas atmosphere, accompanying microbes, storage time and temperature—affect probiotic viability and stability (Heller, 2001; Kailasapathy & Rybka, 1997; Saarela et al., 2000; Saxelin et al., 1999). Factors influencing the viability of probiotics during production, processing and storage are shown in Fig. 17.2.

17.3.4.2 Viability in gastrointestinal transit

In order to get health benefits, an adequate number of probiotics should remain viable in GIT. Challenges encountered by probiotic bacteria following ingestion is low fasting pH (1.5) of the stomach (Waterman & Small, 1998) and high bile salt conditions in the intestine (Kent & Doherty, 2014). The probiotic organism must possess the ability to bear these harsh conditions, to attach to the gut epithelium (Ranadheera et al., 2012; Vasiljevic & Shah, 2008), and to reach the site of action alive and functionally active. Also, it has been demonstrated that probiotic organisms vanish from the GI tract once the ingestion is discontinued (Alander et al., 1999; Donnet-Hughes et al., 1999; Fukushima et al., 1998; Johansson et al., 1998).

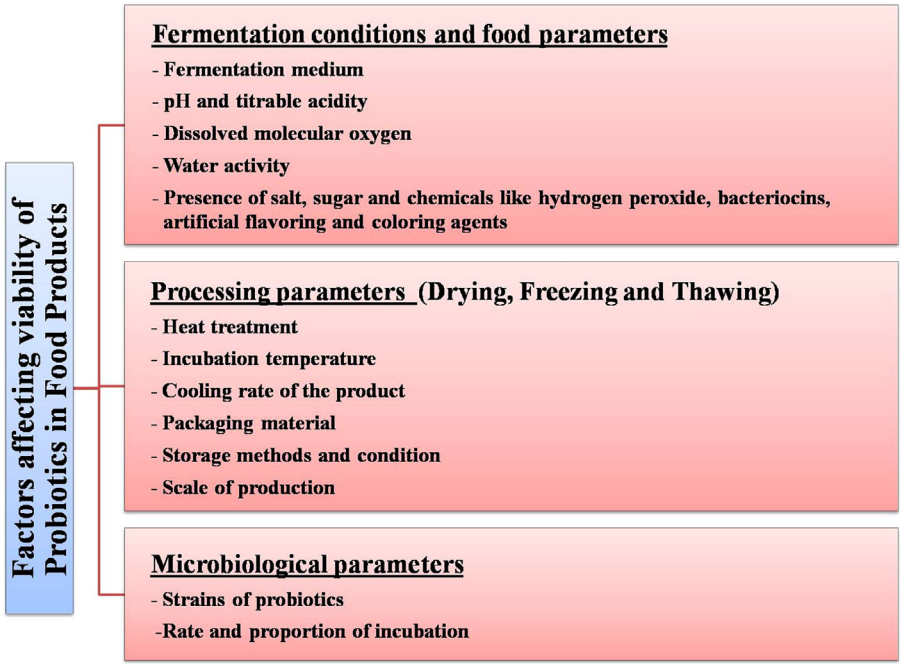

FIGURE 17.2 Factors affecting the viability of probiotics during production, processing, and storage.

17.3.5 Growth and survival in fermented dairy beverages at large scale industrial production

The key factor that needs to be considered for probiotic fermented dairy beverages is the slow growth of several probiotic organisms in nonaugmented/enriched milk from unfavorable production conditions, that is, low fermentation temperature, and generation of off-flavors (Gomes & Malcata, 1999; Østlie et al., 2003; Saarela et al., 2000; Saxelin et al., 1999).

In Japan, the Fermented Milks and Lactic Acid Bacteria Beverages Association have set a minimum standard of 10^7 viable CFU/mL in dairy products to gain anticipated health effects. So, probiotics must be able to grow in the milk and survive in sufficient numbers (Da Cruz et al., 2010; Saxelin et al., 1999) for enhancing the health effects.

Growth in milk: Probiotic *Lactobacillus* and *Bifidobacterium* strains are observed to have poor growth in the milk due to less proteolytic activity, inability to utilize lactose, or special requirements of certain growth factors that are not present in milk (Gomes & Malcata, 1999; Kailasapathy & Rybka, 1997; Østlie et al., 2003; Roy, 2005).

Survival in milk: Relatively little information is being published on the survival of probiotics in nonfermented milk compared with fermented milk products such as yogurt. Because of the high pH values of nonfermented products, *Bifidobacteria*, survive better in nonfermented milk compared to fermented milk. Also, the presence of starter bacteria and their metabolites may negatively affect the stability of probiotics (Kailasapathy & Rybka, 1997). It has been demonstrated in numerous in vitro studies that milk or milk components can protect LAB and *Bifidobacteria* against low pH and also against bile (Charteris et al., 1998; Conway et al., 1987; Fernández et al., 2003; Saarela, Virkajärvi, Alakomi et al., 2006). This can be justified by the buffering effect of milk. Yet during the bile test at pH 7 milk components also protect organisms, which justifies there might be some other mechanism besides buffering action (Saarela, Virkajärvi, Alakomi et al., 2006). Fermented milk products can also protect bacteria in the harsh environment of the upper GI tract.

17.3.6 Good sensory properties

Milk and fermented milk products are the most promising bioactive ingredient delivery vehicles. But several challenges associated with sensory traits and physical stability are encountered during the incorporation of bioactive ingredients into milk beverages. This may render the final product unpleasant for consumers (Shahidi & Alasalvar, 2016). The presence of probiotics in food products should not create any unpleasant flavors or textures. Thus, the main consideration in the development of any food product is that the product should have met the acceptable ranges of consumer needs (Corbo et al., 2014; Shahidi & Alasalvar, 2016).

17.3.6.1 Flavor

While developing functional beverages, the main aim is not restricted to health benefits but extended to good taste, too. Flavor determines the overall acceptability of probiotic foods. While developing new functional milk beverages it is important to know:

1. Nature/characteristic of all the ingredients and microorganisms.
2. Feasible interactions with food components.
3. Effects of process and storage conditions on each functional ingredient.
4. Technological improvements in the process (Allgeyer et al., 2010; Corbo et al., 2014; Esmerino et al., 2017).

During the production of probiotic fermented dairy beverages, inherent off-flavors of certain ingredients in new formulations are challenging. Thus, to reduce or eliminate the off-flavor, it is necessary to know all the ingredients and microorganisms utilized in product manufacturing. For example, when the commonly used functional ingredients, such as glucosinolates and polyphenolics (resveratrol, quercetin, and fisetin), are added to any product, they may introduce undesirable bitter and mouthfeel characteristics to the product (Gaudette & Pickering, 2013). Kawaii (2014) issued a patent on the development of an improved flavor of fermented milk. He employed a lactose degradation step along with the addition of whey powder (WP) to the fermentation base. Thus, balance is maintained between sweet and sour taste.

17.3.6.2 Texture and mouth feel characteristics

Other sensory attributes to be considered are texture and mouthfeel characteristics. Obviously due to low total solid contents in low-fat or fat-free fermented milk resultant probiotic beverages possess unwanted texture, rheology, and sensory features as compared to full-fermented milk. Consumers expect visual and textural properties of fermented beverages equivalent to the properties of traditional drinks. When fermented milk-based beverages are produced using probiotic strains of *Bifidobacterium* spp. and *L. acidophilus*, the product results in poor flavor, texture, and structure (Gallardo-Escamilla et al., 2007; Patrignani et al., 2009).

17.3.7 Maintenance of valuable heat-labile molecules

In the production of probiotic fermented dairy beverages, lactoperoxidase and other heat-labile molecules are expected to be retained. This can be achieved using new and emerging nonthermal technologies.

17.4 Advanced strategies to overcome the limitations associated with dairy-based probiotic fermented beverages

17.4.1 Maintenance of viability and functionality of probiotics

A prerequisite for getting health benefits from probiotics is good viability and functional efficacy (Saarela et al., 2000). Therefore, probiotic products should retain a sufficient amount of probiotic strain during storage and GI transit. In certain instances, probiotic efficacy is related to the metabolic activity of active cells in GIT which leads to the production of effector molecules, e.g., short-chain fatty acids and butyrate. The challenge of delivering viable cells to GIT can be addressed by product formulation and encapsulation (Sarao & Arora, 2017).

17.4.1.1 Enhancing and maintaining probiotic viability and stability during production

The first production step affecting the viability and stability of probiotics is their growth in fermenters.

Immobilization

Immobilization of probiotics in a suitable carrier matrix alters cell physiology, morphology, membrane composition, metabolism, and tolerance to antimicrobial compounds during growth in continuous culture as compared to free-cell batch fermentation (Doleyres & Lacroix, 2005). Additionally, immobilization makes cells more stable and productive (Doleyres et al., 2002).

Use of protectants for probiotic stabilization during manufacturing, free-drying, and spray drying

The viability of probiotic culture during manufacturing can also be improved by the addition of various protective compounds. For instance, to energize cells on exposure to acid, glucose can be included (Corcoran et al., 2005) and

cryoprotectants such as inulin can be added to improve survival during freeze-drying (Carvalho et al., 2004a). Once harvested, cells are freeze-dried, spray dried, or encapsulated to enhance their stability. Survival of probiotics during these steps can be enhanced by the addition of protectants, which can reduce the treatment-induced injuries. A large variety of compounds including skim milk (with or without supplements) and different carbohydrates (such as sugars, fibers) can be used as cryoprotectants for LAB (Carvalho et al., 2004b; Hubalek, 2003; Saarela, Virkajärvi, Nohynek et al., 2006). Few of the above-mentioned molecules can be used as thermo protectants during spray drying.

The probiotic cultures are available in different forms such as freeze-dried/dried form/spray-dried powder for the development of fermented food products (Lievense & Van't Riet, 1993; Holzapfel et al., 2001). Based on the earlier reports, several different strains, including *Lb. paracasei* (Desmond, Stanton et al., 2002; Gardiner et al., 2000), *Lb. curvatus* and *Lb*. sp. 8Z (Mauriello et al., 1999), *Lb. acidophilus* (Prajapati et al., 1987), *Lb. bulgaricus* (Teixeira et al., 1995), *Lb. helveticus* (Johnson & Etzel, 1995), *Lb. rhamnosus* GG (Corcoran et al., 2004) and *Bifidobacterium ruminantium* (O'riordan et al., 2001) were used for the development of fermented food products.

There are some limiting factors of the survival of microbes in the drying process due to temperature and osmotic extremes (Gardiner et al., 2000; Selmer-Olsen et al., 1999; Silva et al., 2002; Teixeira et al., 1995) which leads to the damage of the cell membrane and proteins in the microbes. However, spray drying is the most effective process for the preparation of functional foods. In the meantime, the changes in the probiotic features are also taken into account (Teixeira et al., 1995; To & Etzel, 1997a, 1997b). Hence, the addition of thermos-protectants such as trehalose (Conrad et al., 2000); nonfat milk solids and/or adonitol (Corcoran et al., 2004; Selmer-Olsen et al., 1999); growth-promoting factors, including various probiotic/prebiotic combinations (Corcoran et al., 2004; Desmond, Ross et al., 2002; Desmond, Stanton et al., 2002; Mitsuoka, 1992; Modler et al., 1990); and granular starch (Crittenden et al., 2001) have been employed to enhance the viability of cultures during the drying process. A research report revealed the addition of gum acacia before starting the drying process in *Lb. paracasei* NFBC 338, and it provided a shield to the organism compared with milk powder alone (Desmond, Ross et al., 2002). However, the addition of inulin and polydextrose ended with less impact on the probiotic viability during spray drying or powder storage (Corcoran et al., 2004). The recent research studies reported that the freeze-dried *L. bulgaricus* survived better at 20°C when the cells had been grown with the addition of fructose, lactose or mannose were added to the drying medium (Carvalho et al., 2004a) especially trehalose (de Castro et al., 2000).

Use of encapsulation for probiotic stabilization during manufacturing, free-drying, and spray drying

Various research reports revealed the impact of encapsulation/microencapsulation during the manufacturing, freeze-drying, and spray drying of probiotic strains (Das et al., 2014; González-Sánchez et al., 2010; Menezes et al., 2013; Millqvist-Fureby et al., 2001; O'riordan et al., 2001; Ozyurt & Ötles, 2014; Sheu & Marshall, 1993). Alginate, carrageenan, cellulose acetate phthalate, chitosan, gelatin, gum arabic, and starch (Krasaekoopt et al., 2003) acted as a carrier or supporting material in the encapsulation process; further, various techniques are employed including extrusion, emulsion, and phase separation during the encapsulation process (Kailasapathy, 2002; Kent & Doherty, 2014; Siuta-Cruce & Goulet, 2001).

The immobilized probiotic bacteria (Rao et al., 1989; Sheu & Marshall, 1993), when added into fermented frozen dairy products, showed improved viability of $>10^5$ CFU/g in the product compared to counts of $<10^3$ CFU/g of the nonimmobilized one (Shah & Ravla, 2000, 2004). A research study (Guerin et al., 2003) envisaged that the *Bifidobacterium bifidum* cells, encapsulated in gel beads composed of alginate, pectin, and whey proteins, and surrounded by two membranes, exhibited good survival at pH 2.5 for up to 2 hours, but free cells did not survive. Encapsulation of *Lactobacilli* is using calcium-alginate that enhanced the heat tolerance efficiency of the organism (Selmer-Olsen et al., 1999). Some calcium-alginate beads have shown the prolonged viability during storage of spray-dried *Bifidobacterium ruminatium* (O'riordan et al., 2001). The usage of gum acacia has also played a pivotal role in the stability of dried *Lb. paracasei* NFBC 338 during powder storage at 15°C and 30°C, by up to 1000-fold, and also afforded protection to *Bifidobacteria* (Desmond, Ross et al., 2002; Lian et al., 2002). Further, it has been observed that the strains of *Bifidobacteria*, capable of producing exopolysaccharides, may be naturally protected (Roberts et al., 1995).

Resistant starch encapsulation

Starch is a dietary component having an important role in colonic physiology and functions and a potential protective role against colorectal cancer (Cassidy et al., 1994; Silvi et al., 1999). Due to the nondigestion of resistant starch (RS) by pancreatic amylase in the small intestine, it reaches the colon where it can be fermented by human and animal gut

microflora. RS are classified into three types: RS1, 2, and 3 respectively. Starch entrapped within the food matrix, granular starch, and RS3, that is, retrograded starch formed by food processing (Englyst et al., 1992). It has been demonstrated that the intestinal population of *Bifidobacteria*, *Lactobacilli*, *Streptococc*i, and enterobacteria increased when rats were fed with native potato starch (RS2). The short-chain fatty acids were produced due to the fermentation of carbohydrates by anaerobic bacteria and the pH in the lumen was also lowered (Kleessen et al., 1997; Le Blay et al., 1999; Macfarlane, 1991).

RS is available in some food plants; due to its size and molecular nature they are not completed digested (Vonk et al., 2000). Hence, they reached the colon through the small intestine as fermentable carbohydrate sources for intestinal bacteria (Cummings & Macfarlane, 1997a, 1997b). Reports revealed the increasing trend of *Bifidobacteria* in the animal's intestinal tract (Brown et al., 1997, 1998; Kleessen et al., 1997; Silvi et al., 1999; Wang et al., 1999) due to RS. These resistant starches can also be used as prebiotics (section) besides being a conventional prebiotic, and rendered the viability of probiotic populations from the food to the large intestine. The adhesion to starch by intestinal bacteria may also provide advantages in new probiotic technologies to enhance the numbers of viable and metabolically active probiotics to the intestinal tract (Crittenden et al., 2001). At this juncture, it is important to mention the encapsulate probiotics within starch granules coated with amylose (Myllarinen et al., 2000). A research report stated that a good adhesion and efficient utilization of raw starch is created by bifidobacterial strains (Crittenden et al., 2001).

17.4.2 Strategies used to prevent organisms from oxygen stress

During storage, the viability of *Bifidobacteria* is affected by oxygen content. The redox potential for probiotic bacteria like *Bifidobacteria*, which are anaerobic, makes oxygen toxicity an important and critical problem to be solved. Oxygen affects probiotic cultures in two ways. First, certain probiotics are sensitive to oxygen and die in the presence of it. Second, certain starter organisms such as *Lb. delbrueckii* subsp. *bulgaricus*, in the presence of oxygen, produces peroxide. Also, synergistic inhibitory effects of oxygen and peroxide have been reported (Lankaputhra & Shah, 1996). Therefore, the removal of *Lb. delbrueckii* subsp. *bulgaricus* as starter cultures can improve the viability of probiotics.

Detrimental effects of oxygen can be prevented by the use of antioxidants or oxygen scavengers (Dave & Shah, 1997b; Talwalkar & Kailasapathy, 2003, 2004). Also, highly soluble oxygen can be excluded during the production of probiotic products using special equipment that can provide anaerobic conditions.

17.4.2.1 The use of oxygen scavengers

Ascorbic acid (vitamin C) is a powerful oxygen scavenger. Yogurt fortification using ascorbic acid can also increase the nutritive value. It has been observed that while stored in plastic cups, yogurt shows increasing levels of oxygen and redox potential. But upon the addition of ascorbic acid, the redox potential remains lower (Dave & Shah, 1997b). Aerobic organisms such as *S. thermophilus* would not be able to survive in the presence of ascorbic acid. But the viability of microaerophilic or anaerobic organisms such as *Lb. delbrueckii* subsp. *bulgaricus* is anticipated to advance with increasing concentrations of ascorbic acid. Survival of *Lb. acidophilus* can be improved in the presence of ascorbic acid, but the survival of anaerobic *Bifidobacteria* cannot be improved efficiently (Dave & Shah, 1997b).

17.4.2.2 Addition of cysteine

Cysteine (sulfur-containing amino acid) delivers amino nitrogen as a growth factor and reduces the redox potential. Both of these favors the growth of anaerobic *Bifidobacteria*. Survival of *Lb. acidophilus* and *Bifidobacterium* spp. was determined to improve when providing 250 mg/L of cysteine. *S*treptococcus *thermophilus* shows a different response to cysteine. At the low level (50 mg/L), the growth was found to be promoted because of a slight decrease in redox potential; whereas, at a concentration above 50 mg/L, too much reduction in the redox potential affected bacterial growth (Dave & Shah, 1997). Similar results were found in case of *Lb. delbrueckii* subsp. *bulgaricus*. Also, in the case of *Bifidobacterium* spp., several organisms were found to be increasing even without creating anaerobic conditions when a whey-based medium containing amino acid and yeast extract were used (Dave & Shah, 1997, 1998).

17.4.2.3 Use of oxygen impermeable packaging material

The packaging is the last step in probiotic food production. Oxygen can penetrate packaging material during storage. Therefore, oxygen barrier packaging material such as glass or aluminum foil should be used to encourage the survival of anaerobic probiotic bacteria (*Bifidobacteria*) (Saarela et al., 2000). A research report (Dave & Shah, 1997a) revealed that when organisms are stored in glass bottles and plastic containers, the dissolved oxygen level may play a major role

in the survival of *Lb. acidophilus*. Being an aerobic organism, yogurt starter cultures showed higher numbers when stored in plastic containers than glass. In contrast to that, the survival of *Bifidobacteria* in yogurt stored in the glass is higher than in plastic containers. Also, the survival and viability of *Bifidobacteria* were found to be higher in de-aerated milk (Klaver et al., 1993). Hence, storage of products in glass containers or an increase in the thickness of packaging materials should be promoted to reduce the penetration of oxygen.

17.4.3 Modifications of the composition of the fermentation medium to improve growth of probiotics in milk

Probiotic organisms can grow efficiently in synthetic media such as tryptose peptone yeast (TPY) and de Man, Rogosa, and Sharpe (MRS) broth, as compared to milk (Shah, 2000). However, these media are complex and costly to be used on an industrial scale. It may also generate off-flavor if not thoroughly washed before incorporation. To increase quality, that is, viability, and texture of probiotic products, milk-based media containing casein are preferred. However, the poor and slow growth of probiotic organisms in milk may increase the risk of overgrowth of undesirable microorganisms which may produce off-flavors.

Lactobacillus acidophilus and *Bifidobacterium* spp. demonstrated some level of β-galactosidase activity; their growth in milk-based media is poor. The reason can be the low (insufficient to induce growth) concentration of free amino acids and small peptides in milk. The enhanced growth of *Lb. acidophilus* and *Bifidobacterium* spp. in milk, with the addition of casein and whey protein, hydrolyzates (Desai et al., 2004; Lucas et al., 2004).

17.4.3.1 Use of functional prebiotic ingredients

Prebiotics are a potential source to introduce the beneficial bacteria into the colon through a dietary supplement. The desirable bacteria in the intestinal region stimulated by prebiotics are nondigestible dietary components that pass through the colon (Gibson & Roberfroid, 1995; Van Loo et al., 1999). Due to the potential synergy between probiotics and prebiotics, foods containing a combination of these ingredients are often referred to as synbiotics (Collins & Gibson, 1999; Crittenden, 1999; Gibson & Roberfroid, 1995).

17.4.3.2 Supplementation of milk with nutrients

In yogurt preparation, the predominant *S. thermophilus* in early fermentation hours by the reduction of redox potential and pH favors the growth of *Lb. delbrueckii* subsp. *bulgaricus*. *Streptococcus thermophilus* (Shihata & Shah, 2000, 2002). A special mention here, the production of lactic acid during the refrigerated storage by *Lb. delbrueckii* subsp. *bulgaricus* leads to the loss of viability of probiotic bacteria and, hence, ABT cultures (e.g., ABT-1 and ABT-2) are used to overcome this problem. Researchers (Dave & Shah, 1998) studied the effects of some dairy and nondairy ingredients (WP; whey protein concentrates, WPC; and acid casein hydrolysates) on the viability of *Lb. acidophilus* and Bifidobacteria in yogurt made from four commercial starter cultures (Lucas et al., 2004).

17.4.4 Two-stage fermentation

The production of inhibitory substances such as acid and hydrogen peroxide by starter cultures lead to the poor survival of probiotic cultures. Hence, the problem was encountered by the addition of probiotic bacteria after the fermentation process; however, the low survival of the probiotic bacteria is a question mark in this approach. Hence, another method would be employed to carry out the initial fermentation with probiotic cultures, followed by completion of fermentation with, for example, *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (Lankaputhra & Shah, 1997). By this two-step fermentation process, the period of fermentation may be slightly longer, however, the survival of probiotic bacteria was retained (Tamime et al., 2005).

17.4.5 Applications of direct vat set

Because of the poor growth of probiotics in milk-based media, the production of bulk cultures is a difficult and time-consuming process. In contrast to starter cultures where inoculum size is 1 mL 100/mL, a larger inoculum size of 5−10 mL 100/mL is used in the case of probiotic cultures. Also, probiotic organisms don't grow efficiently if added with other organisms such as *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. Also, the acid generated by starter culture results in poor survival of the probiotic bacteria (Tamime et al., 2005).

For any probiotic product, the prime requirement is that it must have a large number of stable, uninjured, viable starter and probiotic LAB cells. Traditionally, liquid frozen probiotic cultures were used. But later on, freeze-drying and spray drying became favorable methods due to big savings in the cost of transport and storage, and increased culture stability made freeze-drying and spray drying methods more favorable (Champagne et al., 1991; Gölker, 1993). Though spray drying is more economical at an industrial scale as compared to freeze-drying (Gölker, 1993; Johnson & Etzel, 1993), many LAB can not tolerate the relatively high temperature of spray drying (Porubcan & Sellars, 1979). Thus, in contrast to spray drying, freeze-drying is less damaging to organisms (Porubcan & Sellars, 1979) and is a method most commonly used for manufacturing dried LAB. To overcome cell injury during drying and storage, protectants (lactose, glucose, and monosodium glutamate) are added (Champagne et al., 1991; Drake & Lederberg, 1992).

To overcome all the above-mentioned limitations and expenses, an economical alternative that is used is the production of the desired culture at higher concentrations and direct addition of concentrated culture to process milk, that is, direct vat inoculation. Commercially important probiotic organisms are produced in highly concentrated form from direct vat set (DVS) applications (Honer, 1995). This is preferred because of the problems associated with the production of probiotic organisms in bulk at the production site in actual fermenters. This approach has proven to be appropriate for some strains, including *Lb. acidophilus* cultures (Espina & Packard, 1979; Prajapati et al., 1987), *Lb. paracasei* (Desmond, Ross et al., 2002; Desmond, Stanton et al., 2002; Gardiner et al., 2000), and other probiotics including *Bifidobacterium* spp. Lian et al. (2002).

17.4.6 Exploitation of cellular stress response for enhanced technological performance/ biotechnological approaches

The viability of probiotic culture may also be determined by the physiological state of the cultures. Induction of stress responses in strains can considerably affect the survival during processes such as freeze-drying, spray drying, and also during GI transit. Indeed, we have recently generated probiotic cultures that overexpress the heat shock proteins GroESL and have demonstrated improved performance of the culture under a variety of conditions including heat, spray drying, and exposure to gastric acid (Desmond et al., 2004).

The stress tolerance of the probiotic strains at low pH (Lorca & de Valdez, 2001; Maus & Ingham, 2003; Saarela et al., 2004), heat (Ananta & Knorr, 2004; Desmond, Stanton et al., 2002), and drying (Desmond, Stanton et al., 2002; Prasad et al., 2003) had been overcome by the activation of stress response genes in *L. plantarum*, *Lb. paracasei* and *Lb. salivarius* (Corcoran et al., 2006; Derzelle et al., 2003; Sheehan et al., 2006).This acid adaptation study has reported in food pathogens such as *Listeria monocytogenes* and *Salmonella choleraesuis* serotype *typhimurium* (Foster & Hall, 1990; Gahan et al., 1996), and research efforts are carried out regarding the stress response of isolates (Desmond, Stanton et al., 2002; Kullen & Klaenhammer, 1999; Prasad et al., 2003; Shah, 2000; Walker et al., 1999) and the regulation of genes involved in stress mechanisms (Kullen & Klaenhammer, 1999). Oxygen stress is a major stress in probiotic bacteria (Shah, 2000). A protein Osp was expressed in *Bifidobacterium longum* during oxygen stress (Ahn et al., 2001; Boutibonnes et al., 1992). Studies reported increased heat tolerance in *Lactobacilli* (Gouesbet et al., 2002; Teixeira et al., 1994). There have been intensive research efforts carried out into the genetic characterization of probiotic bacteria (Aymerich et al., 1993; Lin et al., 1996; McCracken & Timms, 1999; Wei et al., 1995), especially the availability of full genome *Lb. plantarum*, *Lb. johnsonii*, and *B. longum* (Klaenhammer et al., 2002; Renault, 2002). An effective probiotic strain may come into effect in the future through genetic tools (Iwaki et al., 1990; Kleerebezem et al., 1997; Seegers, 2002; Steidler et al., 2000).

17.4.7 Improvement in growth and survival of probiotics in fermented dairy beverages at large scale industrial production

17.4.7.1 Growth improvement in milk

Growth of probiotic organisms in milk (fermented dairy beverages) can be enhanced by adding various N and C sources, and the addition of other substances such as fructo oligosaccharides (Shin et al., 2000), caseinomacropeptide, WPC (Janer et al., 2004), tryptone (Østlie et al., 2003), yeast extract (Stephenie et al., 2007), nucleotide precursors and iron (Elli et al., 1999); these are all are found to encourage the growth of probiotic bacteria in milk. *Lactobacillus delbrueckii* subsp. *bulgaricus* cell extracts (with β-galactosidase and protease activities) (Gaudreau et al., 2005), tryptone and fructose (Østlie et al., 2003), and inulin, lactulose, raftilose, and Hi-Maize (Desai et al., 2004) have proved useful

growth promoters for probiotic *lactobacilli* in milk. The growth-promoting capacity of different substances depends on the type of probiotic strain, as every strain has a different capacity to utilize lactose and proteins in the milk.

17.4.7.2 Survival in milk

The survival stability of probiotic organisms in milk is determined by the strain, formulation properties, and storage time. Storage for more than 2 weeks reduces viability (Hosono, 1999; Hughes & Hoover, 1995; Martinez-Villaluenga et al., 2006; Saarela et al., 2003; Saarela, Virkajärvi, Alakomi et al., 2006; Sanders et al., 1996). Raffinose family oligosaccharides can improve probiotic storage stability (Martinez-Villaluenga et al., 2006), in a strain-specific manner. Another factor that affects the stability of *Bifidobacteria* in milk is their oxygen sensitivity (Bolduc et al., 2006; Shah, 2000).

17.4.8 Uses of starter culture to improve texture and mouthfeel characteristic

Numerous studies have been performed for improving sensory qualities of beverages either by using exopolysaccharide (EPS)-producing starter cultures or by adding different types of hydrocolloids (Gallardo-Escamilla et al., 2007; Lin & Chien, 2007; Yilmaz et al., 2015). EPS-producing LAB and *Bifidobacteria* are used in the fermentation of dairy products. These organisms can improve texture and reduce the syneresis of end products. Also, some EPS produced by LAB show beneficial impacts on human health (Pv et al., 2009).

17.4.9 Maintenance of valuable heat-labile molecules

For maintenance of heat-labile molecules, using low temperature, micro exposure of pulsed electric field, or combine both, leads to the maintenance of product quality and nutrients (Shahidi & Alasalvar, 2016). The high-pressure homogenization treatment of milk can also maintain the texture parameters of probiotic fermented milk (Corbo et al., 2014; Fernández et al., 2003). It has been demonstrated that the encapsulated strain of *Lb. paracasei* when processed through 50 MPa pressure for five passes, resulted in a high yield of viable cells and even showed high viability till the end of storage in fermented milk (Corbo et al., 2014).

The other two advanced technologies, microencapsulation and nanotechnology have evolved. These can potentially supplement bioactive and nutritional ingredients in fermented dairy beverages. These technologies have already been used and reported (Allgeyer et al., 2010).

17.4.10 Nonviable microorganisms

Though the viability and activity of probiotics are recommended for optimum activity, it has been demonstrated that nonviable probiotic organisms also impart certain health benefits such as immune modulation and carcinogen binding in the host (Salminen et al., 1998, 1999). Thus, in order to have adequate counts of cells for such organisms in the product, it is appropriate to have higher growth in the production process. However, the maintenance of viability during storage is not required. Few experiments have confirmed that beneficial effects can be achieved by heat-inactivated or gamma-irradiated bacteria, isolated bacterial DNA, or even probiotic-cultured media (Dotan & Rachmilewitz, 2005). Lacteol is a commercially available (since 1907) antidiarrheal product in many countries (Makinen et al., 2012). It is comprised of two nonviable strains of *Lactobacilli* isolated from a human stool (Salazar-Lindo et al., 2007).

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Chapter 18

Probiotics in dairy products: microencapsulation and delivery

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18.1 Probiotics: definitions, classification and consumption trends

Probiotics, generally defined as a single or multiple culture of living microorganisms, have been recognized to provide, when supplied to animals or humans, health advantages by strengthening the activity of the gastrointestinal microbiota.

According to the current scenario provided by regulations and products existing on the market, probiotic can be divided into two clusters: probiotic food and beverages (dairy-based probiotics and no dairy-based products), and pharmaceutical probiotic formulates.

As time passed, the definition of probiotic has been revised according to new regulations and evidence based on the use of the term and on required attributes of strains and products.

According to the United Nations and WHO guidelines, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit for the host" (Food and Agricultural Organization of the United Nations & World Health, 2002). Thanks to the advances in the probiotic field, several terms linked to the original concept have been conceived. Table 18.1 provides an overview of terms and definitions.

Safe, functional and technological roles are the basic attributes of microorganisms to be defined as probiotics. However, the probiotic species/strains authorized for use in foods have not a common regulation. National and international rules are defined by countries and intercountry entities. Although the regulation is not sufficiently harmonized among countries, strict rules are defined by the US Food and Drug Administration (US-FDA) and European Food and Safety Authority (EFSA) to approve probiotics, which established the GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety) status, respectively.

The International Scientific Association for Probiotics and Prebiotics provided appropriate consensus statement documents on the definition and scope of probiotics (Hill et al., 2014), prebiotics (Gibson et al., 2017) and synbiotics (Swanson et al., 2020). These documents act as reliable tools to support stakeholders, including consumers, researchers, healthcare professionals, industry, and legislators, with unambiguous guidelines for defining and using each term.

Due to the high growth of functional products, many regulation issues have arisen, especially concerning naming, production system and market related aspects. Moreover, there is not a universal regulation of the probiotics market, which differ from country to country, making it harder to harmonize all regulatory aspects.

In Europe, probiotics and food-associated microorganisms, recognized as living microorganisms, are not included in medical regulations. In accordance with European guidelines, microorganisms for which a history of use are known are categorized as food ingredients. Currently, the European Union (EU) legislation, under the Food Products Directive and Regulation, is the authority invested in the regulation of the framework regarding probiotics and food supplements (de Simone, 2019). However, in Europe, there is no a regulatory status defining the probiotic category. Since the approval of Regulation (EC) 1924/2006 (lastly amended by Regulation (EU) No 1047/2012) on nutrition and health claims made on food, the use of the term "probiotic" is an unauthorized health claim. In addition, any claims regarding prevention, treatment, or cure of a human disease or that imply probiotic activity, are not permitted (https://ec.europa.eu/food/safety/labelling_nutrition/claims). Moreover, the EFSA is the organization in charge for evaluating health claims and is further authorized by the EU. Not all probiotic claims submitted to EFSA are positively evaluated, and most of them

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TABLE 18.1 An overview of terms related to probiotic concept

Term	Definition	Reference
Probiotic	Live microorganisms that when administered in adequate amounts confer a health benefit for the host	Food and Agricultural Organization of the United Nations & World Health, 2002
	Modified the latter definition slightly and defined probiotics as live microorganisms, that when administered in adequate amounts, confer a health benefit on the host	Hill et al. (2014)
Paraprobiotic (or ghost probiotics)	Non-viable microbial cells (intact or broken) or crude cell extracts (i.e., with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal	Taverniti and Guglielmetti (2011)
Psychobiotic	Probiotics that when ingested at adequate amounts, exert beneficial effects on mental health through an interaction with gut microbiota	Dinan et al. (2013)
Parapsychobiotic	Paraprobiotics that yield advantageous effects on mental health	Nishida et al. (2017)
True probiotic	Viable and active microbial cells	Zendeboodi et al. (2020)
Pseudoprobiotic	Viable but inactive cell in the forms of vegetative or spore	Zendeboodi et al. (2020)
Ghost probiotic	Non-viable cell in the form of intact or ruptured	Zendeboodi et al., 2020
Postbiotic	Functional bioactive components (peptides, bacterial enzymes, peptidoglycan-derived neuropeptides and organic acids, for instance, acetic acid and lactic acid) generated during microbial fermentation having beneficial effects on the host	as reported in Collado et al. (2019)
Synbiotic	Synergistic combination of probiotic, beneficial living organism and of prebiotic supplements	de Vrese and Schrezenmeir (2008)

have been rejected so far. The major issues causing the unsuitability of the claims were weakness of the experimental studies (mainly clinical trials), not distinct and defined claims, and food-matrix insufficiently characterized.

According to a new report by Grand View Research, Inc., the global probiotics market is forecasted to reach US $77.09 billion by 2025. The considerable production and consumption of probiotics is complemented by innovations in probiotics by key players among countries such as China, Japan, and India. In 2018, the Asian Pacific area dominated the probiotics industry with a share of 41.7% and is expected to retain its competitive advantage throughout the forecasted period. Moreover, in the past decade, the region has garnered a significant response when it comes to the adoption of probiotics. This is due to the rising levels of health consciousness combined with wider accessibility of probiotic products in this region. Another key observation regarding the consumption of probiotics in Asia Pacific, is the growing popularity of vegetarian probiotic products leading to an increase in consumer vegetarianism.

About product types, probiotic beverages emerged as the largest segment in 2018 with a revenue of US$39.56 billion. The global growth of the segment is favored by an extensive use of cereal-based fermented beverages with probiotic content. In terms of ingredients, the bacteria segment is expected to dominate the market throughout the forecasted period. The increasing use of bacteria to maintain urogenital health/vaginal health is the main force contributing to the growth of the segment. Moreover, increasing attention is given to animal health, which has been fueling the demand for probiotics for animal nutrition. The segment is expected to register relatively faster growth of over 7% throughout the forecasted period (https://www.grandviewresearch.com/industry-analysis/probiotics-market).

18.1.1 Main microorganisms used as probiotics in foods

The microorganisms commonly used for probiotic formulations belong to bacteria groups, specifically to the genera *Bifidobacterium*, and *Lactobacillus* (new taxonomy of the latter has been proposed by Zheng et al. (2020)), although strains belonging to *Enterococcus, Streptococcus, Bacillus* (currently *Weizmannia*, Gupta et al., 2020) genera have been also used. Yeasts have been studied and exploited to a lesser extent, and the species *Saccharomyces boulardii* is mainly

characterized and used at a commercial level. An overview of probiotic commercial products and relative microorganisms are reported in Table 18.2 (Attri, Singh, Kumar & Goel, 2021). It is important to note that according to taxonomic studies, nomenclature of the organisms is under frequent revision. In this chapter, to provide an overview of products and microorganism consistent with the scientific literature, we report the same species names of the original papers.

18.2 Probiotics in foods and beverages

Technologies and innovation in the food and beverage sectors are led by numerous challenges including marketing, technology and most importantly, human safety. The consumers' acceptance of new types of food and beverages is affected by many factors, such as the product processing, as well as cultural, social and lifestyle elements. Consumers have become more aware of the potential health benefits provided by functional foods and beverages. The consumption trend indicates that consumers prefer minimally processed foods and beverages, such as artisan and organic products, which are greener and with low added ingredients and preservatives.

In this frame, probiotics plays a relevant role as healthy products. Although they are well known and linked to dairy-based products, nowadays, the industry offers numerous alternatives to dairy products as well as mixed formulations.

Table 18.2 provides an overview of probiotics available in the world market with a description of the microbial strains and/or of their main characteristics. Next to milk-based category, fruit juice/drink-based products, cereals based and other (Zotta et al., 2020), included in the miscellaneous category, occupy a relevant position, as consolidated and emerging products. In line with consumer demand, which prefers health products with an evident synergy among vegetal and probiotics, the vegetable diet, the animal fat content and lactose intolerance have promoted the development of non-dairy probiotic products.

Commercials non-dairy probiotic products available on the market mainly include those based on fruits, vegetables, cereals, and miscellaneous other matrices (e.g., soy, meat and fish). In addition, the need to promote the consumption of non-alcoholic beverages pushes the industry to design new products, especially for those including functional attributes (La China et al., 2018). To produce beverages with functional features, cereals, fruits, and vegetable appear to be suitable raw materials. Also, mixed products composed of dairy derivates and vegetables matrices arise on the market.

A positive trend emerges from the interest in the development of fruit juice-based functional beverages, supplemented with probiotic and prebiotic ingredients. Fruits and their juices, because of their composition (minerals, vitamins, dietary fibers, antioxidants) and their fermentative attribute due to the presence of sugars, possess optimal attributes to transform into functional beverages. In addition, their basic sensorial profile is appreciated by many consumers.

However, some factors affect the viability and survival of probiotic in transformed juices. Intrinsic parameters, such as acidity, pH, oxygen, water activity, NaCl, sugars, added agents, and toxic compounds produced by microorganisms or added as preservative, all endanger the survival of probiotics. Moreover, processing parameters as well as specific characteristics of the used microbial strain, can also negatively impact the effectiveness of probiotics (Patel, 2017). Among intrinsic parameters, the low levels of pH of fruit juices, strongly affect the functionality of probiotics in the final products, due to the high amount in organic acids. The acidic pH reduces the viability of cells because, the need for increasing amount of energy to maintain the intracellular pH contribute to the ATP deficit for the other cellular functions. Moreover, the functionality and viability of probiotics is compromised by the low content of peptides and free amino acids of juices, which have a protective role on probiotic bacteria (Rovinaru & Pasarin, 2020).

To overcome these limitations, especially those related to the low levels of pH of fruit juices, the use of microencapsulation in polysaccharides and protein-based systems is described as a suitable strategy to preserve, protect, and maintain the viability rate of probiotic cells above the critical threshold of 10^6 cfu/mL and to ensure the controlled delivery in the gastrointestinal tract. Fruit juices have been used as a food matrix to study the effectiveness of microencapsulation on the survival of probiotics versus free bacteria during gastrointestinal transit, at low-temperature storage, as well as implications on organoleptic aspects (Nualkaekul et al., 2012). Innovative strategies to increase the stability and viability of probiotics of fruit juices are being developed. Synbiotic products, based on microencapsulation coupled with the addition of prebioxtics, seems to be a reliable choice. Recently, the functionality of synbiotic microcapsules in protecting the survivability of probiotic cells during processing and storage of fruit beverages has been reviewed. Some promising studies focused on the chemical and microbiological characteristics of substrate, the shelf life and consumer acceptability of these products (Rovinaru & Pasarin, 2020).

In this context, the role of the specific microorganisms cannot be underestimated since the probiotic function is a strain-dependent trait. Then, a comprehensive approach to enhance the stability of fruit juices as functional carrier could take into consideration the selection of microbial strains coupled with the technological aspects.

TABLE 18.2 Not exhaustive list of commercial probiotics foods and beverages obtained by bacterial cultures

Category	Brand/trade name	Description/probiotic culture	Origin
Milk-based	Actimel	Probiotic drinking yogurt with *L. casei* Imunitass	France
	Activia	Creamy yogurt containing *Bifidus* ActiRegularis	France
	Gefilus	LGG products (*L. casei* subsp. *rhamnosus* (*Lactobacillus* GG) (LGG)	Finland
	Hellus	Dairy products containing *L. fermentum* ME-3	Estonia
	Jovita Probiotisch	Blend of cereals, fruit and probiotic yogurt	Germany
	Pohadka	Yogurt milk with probiotic cultures	Czech Republic
	ProViva	Fruit drink and yogurt in different flavors containing *L. plantarum*	Sweden
	Rela	Yogurts, cultured milks and juices with *L. reuteri*	Finland
	Revital	Active yogurt and drink yogurt with probiotics	Czech Republic
	Soytreat	Kefir type product with six probiotics	USA
	Yakult	Milk drink containing *L. casei* Shirota	Japan
	Yosa	Yogurt-like oat product flavored with fruits and berries containing probiotic bacteria (*L. acidophilus, Bf. lactis*)	Finland
	Vitality	Yogurt with pre- and probiotics and omega-3	Germany
	Vifit	Drink yogurts with LGG, vitamins and minerals	the Netherlands
	SOYosa	Soy/oat-based products including a refreshing drink and a probiotic yogurt	Finland
	Snack Fibra	Snacks/bars with natural fibers and extra minerals and vitamins	Spain
Fruit juice/ drink based	Garden of flavor probiotic juice	*B. coagulans*	USA
	Biola	*L. rhamnosus*	Norway
	Valio bioprofit	*L. rhamnosus*	Finland
	Rela by Biogaia	*L. reuteri*	Sweden
	Probi-Bravo Friscus	*L. plantarum* and *L. paracasei*	Sweden
	Valio Gefilus	*L. rhamnosus* GG	Finland
	Danone-ProViva	*L. plantarum*	Sweden, Finland
	Tropicana essentials probiotics	*Bf. lactis*	USA
	GoodBelly	*L. plantarum* 299 v	USA
	Bravo Easy Kit for fruit juice	*L. salivarius*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *Lact. lactis*, *Bifidobacterium*	Switzerland
	KeVita active probiotic drink	*B. coagulans*, *L. rhamnosus*, *L. plantarum*	USA
	Healthy Life probiotic juice	*L. plantarum*, *L. casei*	Australia
	Naked, 100% mango juice with probiotics	*Bifidobacterium*	USA
	Uncle Matt's cold pressed water	*B. coagulans*	Florida
	Harvest soul, probiotic juice	*B. coagulans*	Atlanta
	Oasis, Health break probiotic juice Welo probiotic cold pressed drink	*B. coagulans*	Canada

(*Continued*)

TABLE 18.2 (Continued)

Category	Brand/trade name	Description/probiotic culture	Origin
	Body ecology passion fruit biotic	*L. acidophilus* and *L. delbrueckii*	USA
	Mariani premium, Probiotic prunes, Pressery's organic probiotic soup Love Grace probiotic smoothie, Suja pressed probiotic waters	*B. coagulans*	USA
	Garden of life RAW organic kids probiotic	*L. gasseri*, *L. plantarum*, *Bf. lactis*, *L. casei*, *L. acidophilus*	USA
	KeVita active probiotic drink	*B. coagulans*, *L. rhamnosus*, *L. plantarum*	USA
Cereal based	Welo probiotic bar	*B. coagulans*	Canada
	Pop culture probiotic, Vega one, effiFoods probiotic carebars, Good! Greens bars, PROBAR Meal Bars	*B. coagulans*	USA
	Yog active probiotic cereals	*L. acidophilus*	Germany
Miscellaneous	Vita Biosa 10 +	*Bf. animalis*, *Bf. lactis*, *Bf. longum*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *Lact. lactis* subsp. *lactis*, *Lact. lactis* subsp. *lactis* biovar. *diacetylactis*, *L. pseudomesenteroides*, *S. thermophilus*	Canada
	Welo probiotic ferments	*B. coagulans*	Canada
	Sweet earth natural foods-Get Cultured!tm probiotic burritos + probiotic sugar 2.0, Truth bars, Enjoy life foods, Udi's gluten free, Kevita master brew kombucha Bigelow lemon ginger herb plus probiotic tea	*B. coagulans*	USA
	Brad's broccoli peppers	*B. coagulans*	Pipersville, PA
	Nutrus slim muesli	*B. coagulans*	India
	Something to crow about probiotic muesli	*B. coagulans*	New Zealand
	Macrolife macrogreens superfood bars	*L. acidophilus, L. rhamnosus, L. bulgaricus, Bf. longus, Bf. breve*	USA
	ProDenta	*L. reuteri* Prodentis	Stockholm
	SO Delicious	*L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *L. paracasei*, *Bf. lactis* and *S. thermophilus*	North America and Europe
	Daya	*L. plantarum, L. casei*	Canada
	Kite Hill, almond milk yougurt	*S. thermophilus*, *L. bulgaricus*, *L. acidophilus* and *Bifidobacterium*	USA
	Cocobiotic	*L. acidophilus, L. delbrueckii*	Australia
	Rainbow light probiolicious gummies	*L. sporogenes*	USA

Source: Adapted from Attri, S. N., Singh, Kumar, A., & Goel, G. (2021). Probiotics and their potential applications: An introduction. In Goel, G., Kumar A. (Eds). *Advances in probiotics for sustainable food and medicine. Microorganims for sustainability* (pp. 1–26, 21 Series, Chap. 1). Springer Nature Singapore. *Lactobacillus* (*L.*), *Bacillus* (*B.*), *Bifidobacterium* (*Bf.*), *Streptococcus* (S.)

18.3 Factors affecting probiotic survival in foods

The efficacy of probiotics is strictly correlated to the viability and metabolic functionality of strains. The scientific evidences indicated that the minimum dose of a probiotic strain needed to guarantee survival and temporary colonization of gastrointestinal tract (GIT) is 10^9 live cells/day (daily intake); therefore, a food product should contain this amount to be defined "probiotic" and exert beneficial functions on human host.

Survival and performances of probiotics in foods depend on several factors (Meybodi et al., 2020), including intrinsic features of products (e.g., pH, a_w, oxygen, food ingredients and additives), but also process parameters (e.g., heat treatment, cooling and storage conditions, packaging materials, shelf life), and physiological properties of the strain (e.g., adaptation to food matrices, competition with starter and adjunct cultures for nutrients, stress tolerance, interactions with host microbiota) (Fig. 18.1).

Probiotics are mainly delivered through dairy products (i.e., yogurt and fermented milks; Meybodi et al., 2020) characterized by low pH values (due to fermentation process) and by a more or less prolonged storage at refrigeration temperature. Probiotic strains, therefore, should be able to cope with acid and cold stresses and implement defense mechanisms to ensure viability and functionality. Other foods, such as fruit juices (low pH), frozen desserts and ice creams (freezing) were also tested as probiotic carriers (Flach et al., 2018; Frakolaki et al., 2021a) and may impose damage to the cells because of acid and cold conditions.

Water activity (a_w) also affects the viability of probiotics; strains can be exposed to osmotic stress when large amounts of salt or sugar are added to foods. Osmotic stress, for example, may occur in cheeses and in dried foods. Furthermore, the formulation and preservation of probiotic cultures through spray-drying and freeze-drying processes may impose a significant osmotic pressure to cells, impairing survival and functionality of the strain.

Certainly, one of the main factors affecting probiotic viability is the presence of oxygen, and the values of the redox potential (Eh). Lactic acid bacteria are anaerobe O_2-tolerant microorganisms, while bifidobacteria are obligate anaerobe and, then, more sensitive to aerobic conditions. O_2 may be totally or partially reduced by microbial metabolism and may generate reactive oxygen species (ROS; e.g., superoxide and hydroxide anions) that are toxic to the cells. Most of

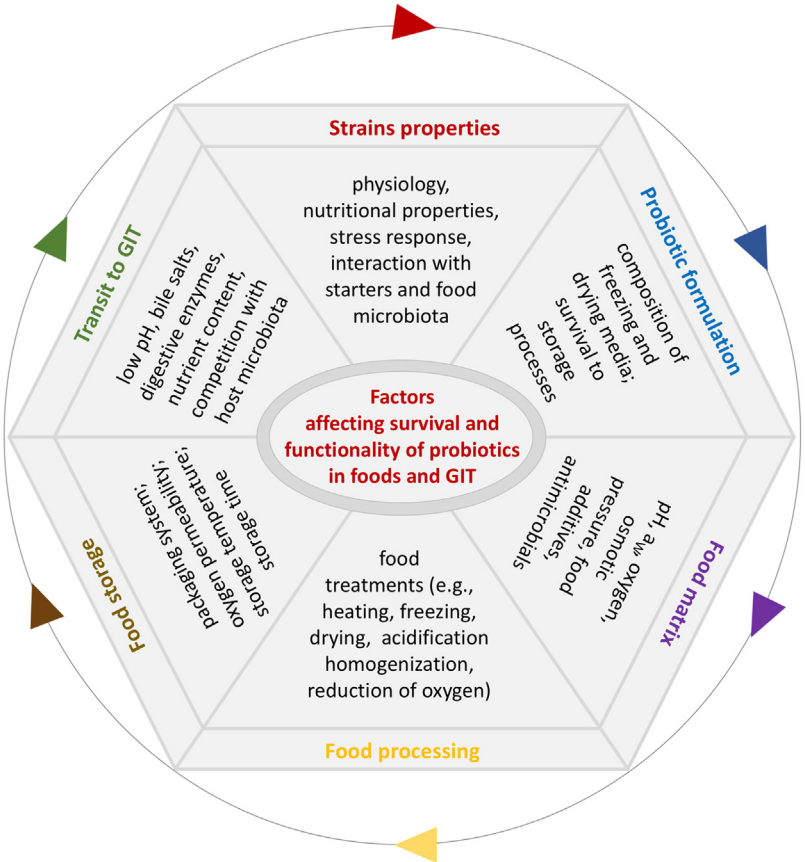

FIGURE 18.1 Factors affecting survival and functionality of probiotics in foods and transit to gastrointestinal tract (GIT).

dairy products are stored and commercialized in packages with high O_2 permeability that could affect probiotic viability during shelf-life. Moreover, during production of yogurts and ice-creams, O_2 may be incorporated in some stages of process (i.e., stirring for yogurts, mixing of ingredients for ice creams) and increase Eh values of product and oxidative stress of probiotic cells. *Streptococcus thermophilus*, the starter culture for the production of yogurt, is able to scavenge O_2 and ROS and, then, may support probiotic survival. Several methods have been proposed to reduce O_2 content in probiotic-supplemented foods (e.g., packaging with limited O_2 permeability, addition of antioxidants or oxygen scavengers), but these strategies certainly increase the cost of products.

Finally, the GIT transit is one of the most crucial steps for the survival of probiotics, and for the exploitation of beneficial functions in human host. Probiotics are exposed to high acid conditions in the stomach, and to proteolytic enzymes and bile salts in small intestine. Moreover, competitive interactions may occur with human colon microbiota (Han et al., 2021).

Like other microorganisms, probiotics develop a general stress response that involves the synthesis of chaperones (e.g., GroES, GroEL, DnaK) and ATP-dependent proteases (es. Clp proteases) that restore the functionalities of proteins and enzymes needed for their metabolic activities. In addition, probiotics can perform more specific defense mechanisms (Amund, 2016; Flach et al., 2018; Mills et al., 2011; Ruiz et al., 2013) that include production of acid shock proteins, proton exclusion through F_1F_0-ATPase, alkalization of cytoplasm via ammonia production (acid stress), small heat shock proteins (heat stress), cold shock proteins, modulation of cell membrane lipid composition (cold stress), accumulation of compatible solutes (osmotic stress), activation of antioxidant enzymes (e.g., catalase, superoxide dismutase, NADH-dependent oxidases and peroxidases; oxidative stress), bile-salt hydrolases, and bile efflux systems (bile-induced stress). Adaptive response to sub-lethal stresses and cross-protection mechanisms may also improve the robustness of probiotic strains in harsh conditions (Terpou et al., 2019).

Stress response, however, is strain-dependent and an accurate characterization of probiotic features and survival tests are needed before incorporating probiotics in food products.

In order to reduce the cell damage imposed by food matrix, production process, and transit to GIT, several compounds that act as growth promoters or as viability protectants (e.g., sugars, vitamins, minerals, prebiotics, skim milk powder, whey proteins) may be added to probiotic-supplemented products. The addition of protective additives, however, may affect the organoleptic features of foods and, therefore, strategies based on carrier systems that do not alter the properties of product are certainly to be preferred.

One of the most studied approaches to improve the survival and delivery of probiotics (but also of other bioactive compounds) is the microencapsulation.

The principles of this technique, as well as the coating materials commonly used for probiotic release in foods, are described next.

18.4 Microencapsulation as strategy to protect vitality and functionality of probiotics

Microencapsulation is a physicochemical or mechanical process, whereby one or more substances (e.g., bioactive compounds, microbial cells) are entrapped and surrounded by a coating material to produce particles (i.e., capsules) whose size varies from a few nanometers to a few millimeters.

According to the size, particles may be classified as macro- ($>5000\,\mu m$), micro- ($0.2-5000\,\mu m$) and nanocapsules ($<0.2\,\mu m$); particle size depends on microencapsulation techniques (see Table 18.3). The entrapped material may be solid or liquid and is, generally, defined as "core"; the coating material may be also referred to as "shell," "wall," "support material," "outer phase" or more easily "capsule." The coating material, moreover, may be distributed around the core both as a mono- or multi-layer structure.

In the past, the term "(micro)encapsulation" was used interchangeably with "immobilization," but it should be specified that with latter technique, the material is mixed and entrapped in a matrix, but is not necessarily enclosed within it, and the size of particles (i.e., beads) are larger than those obtained with microencapsulation.

Microencapsulation technology has several applications, mainly related to the pharmaceutical and health care sector (e.g., drug delivery), but also to personal care, food and beverages, textile, agrochemicals, and construction industries (https://www.grandviewresearch.com/industry-analysis/microencapsulation-market). The global microencapsulation market was expected to expand at a compound annual growth rate of 13.70%, rising to US$19.35 billion by 2025.

Probiotic microencapsulation technology arose in the early 1990s to protect probiotics from the harsh conditions (i.e., low pH, digestive enzymes, bile salts) of the human GIT. Subsequently, this strategy was also extended to the

TABLE 18.3 An overview of the microencapsulation techniques used for probiotic delivery

Techniques	Principle	Advantages	Disadvantages	Coating materials
Spray-drying (capsule size 3–610 μm)	Atomization of core and wall material suspension through a nozzle or spinning wheel, with rapid water evaporation in a dry and high-temperature chamber; capsules are obtained as dried powder	It is the cheapest method, simple operation, stability of the capsules; possibility of adding thermal protectants to improve cell viability	The wall materials must have good water solubility and low viscosity; the high operating temperatures may impair cell viability	Proteins: skim milk powder (SMP, whey protein isolates (WPI), soy protein isolates (SPI). Polysaccharides: alginate, chitosan, gelatin, gum arabic, pectin, modified starch, cellulose acetate phthalate (CAP), maltodextrin
Spray chilling (capsule size 79–83 μm)	Similar to spray drying because produce fine droplets by atomization. Spray chilling, however, is based on the injection of cold air, which enables the solidification of particles contained in a molten matrix	Production of very fine capsules desirable for food applications; high cell survival because does not involve high temperatures	Low encapsulation capacity; possible loss of core material during storage due to polymorphic ri-arrangement of lipid materials during solidification and crystallization process	Polysaccharides: Gum arabic Lipids: Hydrogenated palm oil, sesame oil
Fluid bed drying (capsule size 53–133 μm)	This is a modified spray-drying method and is based on the entrapment of cells into a coating material by using a fluidized bed air drier system (use of an air flow at controlled T °C and humidity)	Economic method; requires low energy consumption	It may cause heat damage to microbial cells; uncontrolled aggregation of particles, due to the coalescence of the wet coated material	Proteins: gluten, caseinPolysaccharides: cellulose and its derivatives (i.e., hydroxypropyl methyl cellulose), k-carragenan, modified starch. Lipids: waxes, fatty acids, oils
Freeze-drying (capsule size 70–800 μm)	Sublimation of frozen solvent under high-vacuum condition (cryodesiccation). The first step requires the freezing of core/wall material mixture	Useful for heat-sensitive compounds and cells; use of cryoprotectants (e.g., polymers, sugars) to improve cell viability	High operational costs; the formation of ice crystals during freezing process and the osmotic pressure during dehydration may impair cell viability	Proteins and amino acids: SMP, SPI, monosodium glutamate. Polysaccharides: alginate (alone or with WPI), maltodextrins. Sugars: fructose, lactose, mannose, trehalose, sorbitol
Emulsification (capsule size 55–2250 μm)	Core and wall materials (dispersed phase) are homogenized with a vegetable oil (continuous phase) to form a stable water-in-oil emulsion; microcapsules are formed under continuous agitation and are collected by filtration or centrifugation	Operational simplicity; high survival rate of microbial cells	Operational costs are high because of the large amounts of vegetable oil required for water-in-oil emulsion; residual oil in the encapsulated material may impair texture and organoleptic properties of foods; not be suitable for the development of low-fat dairy products	Proteins: SMP, sodium caseinate, WPI, chickpea protein. Polysaccharides: Gum arabic, locust bean gum, alginate, k-carrageenan, chitosan, gelatin, maltodextrin, modified starch, CAP, sodium carboxymethyl cellulose
Extrusion (capsule size 15–3500 μm)	The core material is entrapped in hydrocolloid solution, and mixture is extruded under pressure, through nozzles or small opening to form liquid drops that are hardened in a gelling bath	Operational simplicity; low cost at small scale; protection against oxygen toxicity	Difficulty in scaling-up for large industrial applications; capsules size may affect the taste of foods	Proteins: WPIPolysaccharides: mainly alginate beads, xanthan gum, gellan gum, resistant starch, pectin, chitosan, gelatin, poly-L-lysine

(*Continued*)

TABLE 18.3 (Continued)

Techniques	Principle	Advantages	Disadvantages	Coating materials
Complex coacervation (capsule size 10–3000 μm)	The core material is emulsified with a wall solution; a coagulant substance or a solvent is added to reduce the solubility of wall material and to drive the microcapsule formation. Hardening may be achieved through heat treatment or by using a cross-linking agent	Mild operating conditions and low damage rate to the core material; high encapsulation efficiency; good release control properties	High operational cost; large amounts coagulants consumed; chemical residues during processing; not widely applied in food industry	Proteins: milk protein, WPI, gelatin, albumin, egg proteins, lipoproteins. Polysaccharides: Gum arabic, pectin, alginate, xanthan gum, k-carrageenan
Layer-by-Layer(capsule size 47–130 μm)	The layers merge spontaneously (self-assembly) to form stable molecular aggregates through non-covalent interactions (e.g., electrostatic attraction, hydrogen bond, and coordination bond)	Good control of size, shape, composition and thickness of capsule structure, even for very small scale)	The LBL assembly process can take a long time when multiple wall materials with different charges are needed	Polysaccharides: alginate, chitosan, carboxymethyl cellulose.
Electrospray (capsule size 0.3–600 μm)	Transformation of a polymeric fluid in fine droplets through a high voltage electric field. After evaporation of solvent, the polymer particles are collected through a collector to obtain the microcapsules	Formation of very fine and stable microcapsules; operational process under mild and non-thermal conditions; widely used for pharmaceutical application	Distance between the nozzle tip and collector is crucial to obtain correct capsule size and avoid aggregation before solvent evaporation	Proteins: casein, whey protein concentrate (WPC), WPI, SPI, collagen, egg albumen, zein, casein. Polysaccharides: starch, cellulose, pectin, guar gum, chitosan, alginate, k-carrageenan, xanthan, dextran, cyclodextrins

inclusion of probiotics in foods, with particular focus on the stresses related to the raw material, as well as to food production and storage processes. For food applications, the capsule size and materials should be suitable either to protect probiotics and to avoid negative effects on the sensory and texture properties of the final products; aseptic conditions should be maintained throughout the overall process, including sterilization of encapsulation materials and equipment, to prevent microbial contamination.

Today, many microencapsulation approaches, based on different physical and/or chemical principles, have been investigated and tested for the delivery of probiotics in foods, and an overview of the most commonly used techniques are reported in Table 18.3.

18.5 Coating materials for probiotic delivery in foods

The coating materials used for probiotic delivery in foods must meet quality and food safety standards (i.e., food-grade, GRAS status, high hygienic levels, recognized as food additive, edible) and should be selected on the basis of microencapsulation methods, probiotic microorganisms, and food properties. Additionally, they should be economical and should possesses rheological properties (e.g., viscosity, elasticity, and plasticity), such as to ensure good workability and ability to obtain capsules, with adequate features (e.g., cell protection, lack of chemical reactions with core material, barrier to harsh environments). The arrangement of shell matrix on the surface of the core material, in fact, is crucial for the functionality of microcapsules.

Actually, the coating materials for probiotic encapsulation include polysaccharides, proteins and lipids (Abd El-Salam & El-Shibiny, 2015; de la Cruz Pech-Canul et al., 2020; Frakolaki et al., 2021a; Gbassi & Vandamme, 2012; Liu

et al., 2020; Pavli et al., 2018; Yang et al., 2020; Yao et al., 2020 for reviews). Polysaccharides and proteins, due to their hydrophilic nature, provide a good barrier to gases (e.g., O_2, CO_2) but not to water, while lipids being hydrophobic are suitable for gas protection.

Polysaccharides may be natural or synthetic, and may have different charges (i.e., anionic, cationic, non-ionic). The following polysaccharides are reported as the main exploited compounds.

Alginate is a linear polymer (acid α-L-guluronic and acid β-D-mannuronic linked by β(1−4) glycosidic bonds) extracted from the cell wall of brown algae *Laminaria* spp., widely used for probiotic encapsulation, mainly with extrusion and emulsion methods (i.e., sodium-alginate beads). Alginate melts at 60°C−80°C and is insoluble in acid media; however, its porous structure often does not provide an efficient cell protection against the acid stress.

Carrageenan is a linear polymer extracted from red seaweeds (D-galactose linked by α(1−3) and β(1−4) bonds), commonly used as food additive. Although different types (k, ι, λ) of this polymer are available, the k-carrageenan is mostly used for probiotic microencapsulation. k-carrageenan dissolves at 60°C−80°C and its gelation occur at temperatures lower than 40−45°C; capsule formation require monovalent ion environment, such as solution of KCl. k-carrageenan capsules have been found very effective in protecting probiotics in GIT conditions.

Chitosan is a cationic polysaccharide resulting from partial deacetylation of chitin. Its solubility is pH-dependent and at values higher than 5.4 is water-insoluble. Chitosan forms a semi-permeable coating and has been used in combination with other polymers (e.g., alginate, starch, whey protein isolate, xanthan gum) to encapsulate and protect several probiotics under simulated GIT conditions. Alginate-chitosan mixtures reduce porosity of pure alginate beads and increase survival to low pH.

Pectin is an anionic polysaccharide extracted from plant cell walls (e.g., fruit pulp and peels). Obtaining pectin beads is similar to that of alginate ones (by extrusion and emulsion methods with calcium-induced cross-linking), but pectin is more resistant to acid conditions and, compared to alginate, may provide a better protection to GIT and foods with low pH values.

Xanthan and gellan gums are exopolysaccharides of microbial origin (from *Xanthomonas campestris* and *Sphingomonas elodea*, respectively) used in combination with other polymers (e.g., alginate, k-carrageenan, but also casein and derivates) to reinforce the structure of coating material. Gums improve robustness and rheological properties of capsules enhancing protection against GIT and bile salts. Locust bean gum, gum arabic and guar gum have been also used and mixed with alginate or k-carrageenan to develop gel beads or capsules.

Resistant starch (RS) is a type of starch with a marked resistance to digestion by pancreatic enzymes. When RS is mixed with alginate provides a synergic effect on capsule gelation and acts as prebiotic agent; starch may be fermented by colon microbiota providing energy and compounds useful for cell viability. Maltodextrins and cyclodextrins may be produced from starch degradation and also used as coating materials.

Natural polysaccharides are often sensitive to acid and string ion conditions; therefore, some polymers of chemical synthesis have been used to improve stability and structure of microcapsules. Cellulose acetate phthalate (CAP) is a synthetic compound derived from cellulose, which in turn is one of the most important organic polymers. CAP is insoluble at pH below 5, making it suitable for probiotic encapsulation because it does not dissolve in the stomach, but only gradually in gut, releasing the beneficial microorganisms.

Carboxymethyl cellulose and carboxymethyl chitin are also two semi-synthetic anionic polysaccharides used to provide better stabilization and protection of encapsulated probiotic in harsh conditions.

Besides the polysaccharides described above, other polymers (Liu et al., 2020) are gaining interest as coating material for microencapsulation processes, and many studies are addressed on the use of prebiotic substances (e.g., inulin, fructans, fructo-oligosaccharides, galacto-oligosaccharides *psyllium* polysaccharide), bioactive compounds (e.g., lentinan, arabinoxylan) and polymer from agri-food wastes (e.g., fruit wastes).

Proteins used for microencapsulation may be of both animal and vegetable origin, even if those obtained from dairy production are the most exploited. Proteins, however, are mainly used in combination with other coating agents (e.g., alginate, k-carrageenan, pectin).

Whey proteins may be classified in whey protein concentrates (WPC) and whey protein isolates (WPI), with 35%−85% and >95% protein content, respectively. Properties of WP-based capsules are different because of different chemical compositions of WPC (high levels of lactose and total lipids) and WPI (high levels of proteins, low content of lactose and lipids). WP are mainly used with extrusion, emulsion, and spray-drying methods, alone or in combination with other polymers (i.e., alginate, k-carrageenan, pectin, vegetable oils; Abd El-Salam & El-Shibiny, 2015).

Regarding the milk proteins, native casein and its derivate sodium caseinate (SC) are mainly used as a coating material for probiotic encapsulation. Milk proteins have good gelation properties, ability to interact with other polymers to generate complexes, buffering capability that provides an efficient protection to acid conditions, and a good resistance

to heat denaturation. Milk proteins have been successfully used to protect probiotics under GIT conditions and in foods with low pH, such as yogurt (Abd El-Salam & El-Shibiny, 2015).

Gelatin (derived from partial hydrolysis of collagen) has been also used, alone or in combination with other compounds, as a coating agent in extrusion, complex coacervation, spray drying, and lyophilization methods.

Proteins of animal origin, although having satisfactory rheological properties, are inappropriate for encapsulating probiotics in products intended for vegetarian and/or kosher diets.

Proteins from vegetable sources derive mainly from cereals and legumes, such as corn (zein), wheat, pea and soy. Soy proteins isolates (SPI), and, having good emulsifying and gelling properties, are used in several processes (e.g., extrusion, spray drying, coacervation); SPI, moreover, are high quality proteins, used as an alternative for vegetarians, kosher, and subjects with milk intolerances.

Lipids are polymers with low polarity, used as coating materials mainly for reducing or blocking the moisture transport. Generally, lipids are blended with other coating agents (e.g., polysaccharides, proteins) to improve their structure and coating features. The lipids used in microencapsulation may be both of animal (e.g., butter, fish oil, pork oil) or vegetable (corn, sunflower, palm, olive oils) origin. The most used lipids are mono-, di- or triglycerides and, therefore, their properties depend on the composition of fatty acids present in their structure. The solidification of fats is obtained at a temperature below their melting point.

Phospholipids are also used in the food industry for their emulsification capability and for their ability to form micelles and liposomes; the latter are employed as delivery systems of bioactive compounds, but today are not yet exploited for probiotic strains.

An overview on the use of polysaccharides, proteins and lipids in microencapsulation methods are reported in Table 18.3.

18.6 Use of microencapsulation for dairy products

Microencapsulation has been studied and exploited to improve probiotic survival in dairy products, especially in yogurt and fermented milks. Several data, however, are also available for cheeses, desserts and ice cream. Although the microencapsulation of probiotics in dairy products is under investigation for some time (Champagne & Fustier, 2007), the more recent data are reported in this chapter.

Probiotic yogurt, generally contained starter cultures (i.e. *Streptococcus thermophilus*, *L. delbrueckii* subsp. *bulgaricus*) and strains belonging to the species of *L. acidophilus* and *L. casei* groups, and to *Bf. bifidum*, *Bf. lactis* and *Bf. longum* (Frakolaki et al., 2021a). Probiotic viability (10^8 cfu/g) in yogurt should be stable up to 4 weeks at refrigerated temperatures. Microencapsulation, then, is crucial to improve survival during cold storage, and several data indicated that it may result in a lower post-acidification process. Different techniques (i.e., extrusion, spray drying, freeze drying, emulsification) and coating materials (i.e., skim milk, sodium alginate, chitosan, k-carrageenan) have been used (Frakolaki et al., 2021a).

Recently, Frakolaki et al. (2021b) encapsulated (extrusion and emulsification methods) *Bf. animalis* subsp. *lactis* in sodium alginate beads blended with glycerol or k-carrageenan and/or inulin, and evaluated its survival in yogurt. Microencapsulation was effective in probiotic protection and, after 30 days of refrigerated storage, the strain maintained a good level of viability (3 log cycle reduction) compared to encapsulated cells used for control yogurt (5 log cycle reduction). The survival of microencapsulated *L. acidophilus* (sodium alginate coated with xanthan and/or whey proteins) was improved (up to 2 log cycles) during yogurt storage, compared to the viability of free cells (Khorshidi et al., 2021); robustness to simulated GIT was also boosted (up to 3 log cycles). *Lactobacillus acidophilus* microcapsules, moreover, improved texture features (e.g., firmness, adhesiveness, viscosity; decrease in syneresis) of yogurt. Afzaal et al. (2019a) investigated the survival of *L. acidophilus* ATTC4356 in yogurt and simulated GIT with and without microencapsulation in sodium alginate and carrageenan. After 28 days of storage, the viability of encapsulated ATTC4356 decreased of 1.7 log cycles, while that of free strain dropped to 4.8 log cycles. *Lactobacillus acidophilus* beads increased viscosity and reduced the syneresis of yogurt compared to free cells. The survival to simulated GIT was also higher for encapsulated cells. Recent studies show that viability and growth of probiotics in dairy products increased when they were co-encapsulated with prebiotics (Rashidinejad et al., 2020). For symbiotic dairy products, inulin, b-glucan, resistant and waxy starch have been mainly used and incorporated in coating materials. Silva et al. (2018), for example, demonstrated that microbeads of alginate and gelatin (AG) or alginate, gelatin and fructo-oligosaccharides (AGF) improved the survival of *L. acidophilus* during 28 days of storage in yogurt; reduction of cell viability in AG and AGF beads was about one cycle, while that of free cell was about two cycles.

As for yogurt, the species added to probiotic cheeses mainly belong to the genera *Lactobacillus* and *Bifidobacterium*. Emulsion, extrusion and spray-drying are the most used techniques, while skim milk, sodium alginate and k-carrageenan are the common materials (Frakolaki et al., 2021a). Microencapsulated strains were tested for different cheese products. Amine et al. (2014) demonstrate that microencapsulation of *Bf. longum* 15708 in alginate beads (extrusion and emulsion methods) increased viability of the strain during cheddar manufacture and ripened as well as during simulated GIT conditions (up to two cycles compared to free cells). Successively, Afzaal et al. (2019b) investigated the survival of *Bf. bifidum* ATTC29521 in cheddar cheese, in both free and entrapped forms (sodium alginate and carrageenan as wall material), confirming the beneficial effect of microencapsulation. Moghanjougi et al. (2020) prepared cellulose biofilm containing free and microencapsulated (sodium alginate, pectin) cells of *L. acidophilus* and *Bf. animalis* and verified their effect on chemical properties and shelf life of white-brined cheese during 45 days of storage. *Lactobacillus acidophilus* showed higher survival than *B. animalis*, and sodium alginate (SA) provided a better protection compared to pectin. SA-encapsulated *L. acidophilus* had also a high inhibitory effect on *Aspergillus niger*. The type of strain and microcapsule did not affect the chemical properties of cheese. More recent, Neto et al. (2021) prepared a Reno-like cheeses containing *L. acidophilus* LA-3 in both free and microencapsulated form (addition of cysteine hydrochloride and/or ascorbic acid), demonstrating that microcapsules provided a highest viability of probiotic strains during ripening. Whey protein isolate and arabic gum were used instead as wall materials to encapsulate the probiotic *L. plantarum* ATCC 8014, alone or in combination with phytosterols (spray-drying and freeze-drying methods), and to verify the survival of the strain in Iranian white cheese (Sharifi et al., 2021). Co-encapsulation with phytosterols provided the highest viability level compared to free and single microencapsulated cells. Microencapsulation of probiotics was also investigated in fresh cheeses. Lopes et al. (2021), for example, investigated the effect of *L. acidophilus* La-05 in both free and microencapsulated form (with alginate or alginate coated with chitosan) on the quality of spreadable goat Ricotta cheese during 7 days of storage at 7°C. Microencapsulation resulted in higher probiotic survival and improved technological properties of the Ricotta cheese. Encapsulated *L. acidophilus* LA-05, moreover, was also more robust to simulated GIT conditions.

Desserts and ice cream provide a more favorable environment than yogurt and cheeses due to the higher pH and a_w values and the absence of competing microorganisms. Some ingredients, moreover, are rich in fats (e.g., chocolate) and further contribute to strain protection. In these products, however, probiotics may suffer osmotic and freezing stresses and, therefore, microencapsulation may be beneficial. Afzaal et al. (2019b) investigated the survival of *L. acidophilus* ATTC4356, also in ice cream, in both free and microencapsulated form (sodium alginate and carrageenan were used as coating material). After 120 days of storage at −20°C the viability of ATTC4356 decreased 3 log cycle, while the encapsulated strain had higher robustness, showing a cell reduction of only 1 log cycle. Similarly, the survival to simulated GIT was higher for protected cells. Among coating materials, sodium alginate exhibited the better release properties. Successively, Afzaal et al. (2020) evaluated the robustness of *L. casei* in ice cream, using calcium alginate and whey protein concentrate as shell materials. Results confirmed the effectiveness of microencapsulation in viability maintenance (the reduction of only 1 log cycle was observed after 80 days of storage). Moreover, encapsulated strain positively affected the physicochemical and sensory properties of products (e.g., decrease in pH, increase in viscosity). The probiotic *L. casei* Shirota were microencapsulated with different combinations of arabic gum , maltodextrin (M) and reconstitute skim milk (RSM) and inoculated into pudding (Gul, 2017); RSM and combinations of RSM and arabic gum provided the best protection for the probiotic strain. Talebzadeh and Aharifan (2017) developed jellies containing *L. acidophilus* LA-5 in free form, or encapsulated in alginate beads and chitosan-coated ones. Microencapsulation protected the strain against low pH and high temperature, resulting in a greater survival compared to free cells. Chitosan-coated beads provided the best protection and did not impaired product features.

Scientific data suggest that microencapsulation is a prominent tool to improve survival of probiotics in dairy products and GIT conditions. However, further investigations on its effect on strain functionalities are needed to bring microencapsulation from laboratory to industrial scale.

18.7 Challenge and future prospective

Probiotics are recognized as a prominent sector of foods and beverages industry. In recent years the market started to offer several new products differing from the traditional ones for the raw materials, the processing, and the healthy claims.

In this scenario, to satisfy consumers demand, main stakeholders (industry, researchers and regulators) face the need to comply basic requirements as well as new issues arising from products/processes innovation.

The continuous search for new robust strains having peculiar traits, and the effectiveness of the applied technology also to different raw materials respect to dairy based ones, is one example of the complex probiotics area. Moreover, the survival and functionality of probiotics remain a main issue. From the technological point of view, delivery and *in situ* vitality of probiotic products, is under continuous investigation. Microencapsulation of probiotics in foods at an industrial scale, can be a powerful and beneficial technology, albeit with several microbiological, technological and economical challenges to address. Although further studies are needed to improve the effectiveness of microencapsulation technology, the current knowledges highlight its high potential in delivering viable probiotics cultures.

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Chapter 19

The effect of innovative processing technologies on probiotics stability

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19.1 Introduction

The word probiotic has been originated from combination of Greek and Latin word where *pro* means for and *biotic* means life. Probiotics are living and beneficial microorganisms that provides healthcare benefits to the host after consuming an adequate amount. Probiotics were initially taken as "Organisms and substances that positively impact on intestinal balance". Probiotics are live beneficial microorganisms given to improve microbial balance, especially in the gastrointestinal tract. In several research on fermented milk products and the human gastrointestinal tract, the viability and sensitivity of probiotic bacteria has been commonly considered. Now probiotics represents an ever-growing multi-billion-dollar industry and is commonly in use with or without doctor's recommendation in many countries (Boyle and Tang, 2006). Mostly, probiotics (mostly *Bifidobacterium* and *Lactobacillus* strains) are taken from the intestine of healthy human and can also be taken from dairy items. Probiotics also include some of *Streptococcus, Bacillus, Enterococcus* and the (yeast) *Saccharomyces* species; they have been in use as probiotics doe several years . That is why the probiotic strains must be selected on the basis of their suitability for industrial production on a wider range, and also on the basis of their liability to stay live and sustain their properties during manufacturing and deposition (in frozen/dehydrated cultures). They can sustain their life in food manufacturing processes, or in the food products, where it is eventually produced. The populations of 106×10^7 CFU/g in the end item is taken as remedial dose of probiotic culture in highly processed products (Talwalkar et al., 2004) it Heidebach et al. (2010) making 108×10^9 CFU, supplied 100 g/100 mL on regular intake of food, thus providing benefit to consumer overall health. A probiotic product can be referred as a prepared item having probiotic microorganism in an appropriate matrix and in a reasonable amount (Saxelin et al., 2003). In fact, the culture of probiotics works by increasing the production of beneficial microbes in the gut and thus boost the effectiveness of the body's own system by combating with harmful bacteria present in the human body. Probiotics are commonly used in supplements, food, infant formula formulations, and medical devices. The amount of available probiotics usually gets reduced whenever culture is subjected to some harsh environment, such as acidity in the stomach, oxygen, hydrogen ion and presence of antibiotics or any antibacterial material. Using probiotics in the food industry has launched more than 600 dairy products. Probiotic foods are estimated to make up 60%−70% of the overall market of functional products . They have shown remarkable potential as therapeutics for various ailments, mainly GI ailments i.e., infectious diarrhea, antibiotic associated diarrhea, ulcerative colitis, colon cancer, IBS (irritable bowel syndrome), functional GIT problems/necrotizing, enterocolitis and also intestinal disorders, that is, hepatic, encephalopathic. That means their feasibility and functionality of metabolism should be kept up in all operations involving food production, from the very first step of preparation up to its ingestion by consumer; and also, they must get through the gastrointestinal tract alive (Granato et al., 2010). By enhancing the composition of intestinal microbes and enhancing mucosal pathogenic defenses, these bacteria have many benefits for consumer's health (Boylston et al., 2004). Antimicrobial function, antidiarrheal activity, ease from symptoms arising from lactose intolerance, antimutagenic, cancer fighting, boosting immune system, betterment in urogenital health to relief from constipation are some of the most beneficial health effects linked to probiotic consumption. A well-designed strategy

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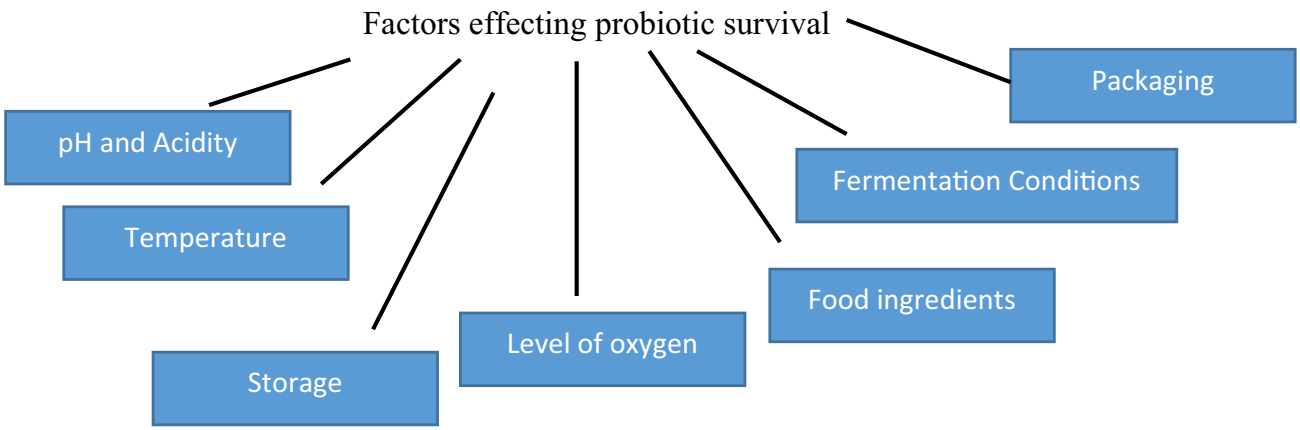

FIGURE 19.1 Factors affecting the survival of probiotics.

(formulation methods, packaging, stability and organoleptic consistency issues) for the introduction of probiotic microbes into the foods is a significant step involved in the production of variety of functional foods. Various factors contributing to functional food quality that are produced on the interaction between prebiotics and probiotics need to be overcome. These factors include deprivation of a knowledge to main factors effecting viability of probiotics, their sensitivity and/or overall performance. In order to attain this objective, prebiotics, with the combination of tailored manufacturing procedures, impact positively on probiotics overall performance and play a vital role in the advancement of viability and stability of probiotic cultures within food during processing and storage. The probiotics survivability in food products is affected by major factors that include pH and postacidification in fermented products during storage. The other factors are production of hydrogen peroxide, change in temperatures during storage, compatability with the end product, insufficient proteases, viability with conventional starter culture bacteria in time of fermentation, etc. Thus, the protection of living probiotics cells became a serious issue. Probiotics are protected in an effective encapsulation system to prevent the cells from damage, to improve shelf storage life and to provide protection from acidic environments (Fig. 19.1).

19.2 Factors affecting the survival of probiotics

19.2.1 Fermentation conditions

Fermentation temperature is one of the significant elements that influence the reasonability of probiotic microbes and other subjective boundaries of probiotic aged items. The optimum temperature for the growth of most probiotics is in the range of 37°C−43°C (Boylston et al., 2004).

19.2.2 Freezing and thawing operations

Probiotic microbes can endure a more extended term in solidified products. The viability of probiotics effected because probiotics get injured during freezing due to mechanical anxieties of the ice precious stones framed in the outside zone or inner layer of the phones, consequently making lethal injury them.

19.2.3 pH and titratable acidity

The viability of probiotics decreases during storage that is influenced by pH and titratable acidity of the products (Mortazavian et al., 2010).

19.2.4 Oxygen content and redox potential

Oxygen content and redox potential are one of the significant factors influencing the survival of probiotics especially during the storage period.

19.2.5 Storage temperature

The survival of probiotic microorganisms during storage is negatively related to storage temperature.

19.2.6 Packaging aspects

The packaging plays a significant role in maintaining the probiotics food quality. The package is an integrated part of the preservation system and functions as a barrier between the food system and the external atmosphere .In this context, probiotic strains are anaerobic and microaerophilic ones. So, the oxygen level in the package during storage should be as low as possible in order to avoid probiotic death.

19.2.7 Food ingredients and additives

Food ingredients can be defensive, unbiased, or impeding to probiotic durability; therefore, the common characteristics of probiotics with various food fixings assumes a significant part in their endurance.

19.2.8 Effect of nonthermal processing techniques on probiotics viability

The incidence of chronic diseases, that is, respiratory disorders, diabetes, cancer and cardiovascular diseases is increasing every day. These diseases are directly related to lifestyle, diet and tangible actions. The exact analysis of these diseases is very complicated and prevention is dependent upon deep-rooted practices to healthy eating and continuous physical activity (Mendis, 2016). In this context, the scientist has been introducing new emerging techniques to improve the health of people by providing the quality of food products (Neuhouser, 2019). Functional foods are helpful to fight against the incidence of these chronic diseases. Probiotics and prebiotic-based functional foods are gaining attention in the market due to their positive health benefits (Santeramo et al., 2018). Probiotic-based functional foods exert several health benefits, that is, improve gut health, boost up immunity, prevention from diabetes, cancer and cardiovascular diseases (Guimarães et al., 2020). Despite all the health benefits of functional food, these products are being rejected by the consumer due to poor taste and inconvenience (Bleiel, 2010). In recent years, efforts are being made to develop functional food with better taste, quality, safety, wellness, shelf life and convenience (Guimarães et al., 2020). Based on these facts, nonthermal emerging technologies are being introduced to develop healthier functional foods. Some of the emerging nonthermal techniques are discussed below (Fig. 19.2).

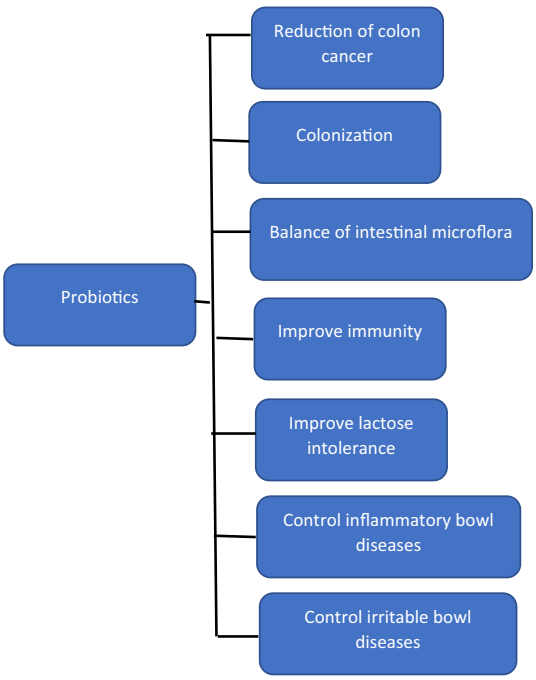

FIGURE 19.2 Health benefits of probiotics.

1. High-power ultrasound (HPUS)
2. High-pressure processing (HPP)
3. Pulsed electric field (PEF)

Therefore, the major aim of this chapter is to assess the effects of emerging nonthermal techniques, that is, HPUS, HPP and PEF on functional products developed with probiotics.

19.2.9 High-power ultrasound

HPUS has been introduced for the enlargement of probiotic and prebiotic dairy-based products. The ultrasound-treated probiotic dairy products have improved probiotic survival, viability, enhanced fermentation activity and increased the production of peptides. The product quality increased due to the production of enzymes and carbohydrate hydrolysis. The result of HPUS on the viability of probiotic products depends on the intensity of power/frequency and duration of sonication processing. Moreover, it also depends on probiotic strains, because different strain behave differently in response to ultrasound because of dissimilarity in their cell structure and composition (Huang et al., 2017). Application of the ultrasound effect increases or decreases the proliferation and endurance of probiotics in food items (Abesinghe et al., 2019) to injured cell membrane and the formation of the pore (Ewe et al., 2012). Mortazavi and Tabatabaie reported these damages, that is, cavities, microvoids, cracks, shrinkage, pores and raptures in cell membrane that directly affect probiotic viability. A method sonoporation is defined as the "production of pores/cavities in the cell membrane" through sonication. Application of sonoporation at a modest level, facilitate the mass transfer across the membrane, improves permeability, supply nutrients and excretion of left overs of cellular metabolism (Ojha et al., 2017; Pitt & Ross, 2003). So, the major phenomena behind sonication have been underestimated and it is microstreaming the microorganisms instead of any physical damage to the cell but clear the way for the movement of substances, gases and nutrients (Dahroud et al., 2016). Sonoporation at high levels causes cell death, cell leakage due to physical damage and changes in the cell membrane (lipid peroxidation) (Ojha et al., 2017). So, it is important to adjust the ultrasound conditions precisely to achieve the desire results (cell permeability) in probiotic food products to avoid cell death. It is worth mentioning that the application of ultrasound in fermented milk products increases the viability of probiotic and shortens the fermentation time that results in more manufacturing of organic acids (Barukčić et al., 2015; Huang et al., 2019; Nguyen et al., 2009). The reason behind this is the modification in bacterial cell composition, releasing β-galactosidase and stimulating the lactose hydrolysis and transgalactosylation during fermentation (Nguyen et al., 2009). Results also revealed that ultrasound-treated fermented milk had improved nutrition, organoleptic characteristics, bioactive peptides and higher concentrations of oligosaccharides (Barukčić et al., 2015). The resulted probiotic-based fermented milk product has low lactose content, higher oligosaccharides, less manufacturing time, enhanced probiotic viability, products with decreased production of undesirable taste (acetic acids and propionic) and reduced ingredients/additive cost (Nguyen et al., 2012). The ultrasound-treated probiotic products have better taste, color, flavor and physicochemical properties similar to pasteurized milk, with the benefit of less processing times (Table 19.1) (Arvanitoyannis et al., 2017) (Fig. 19.3).

19.3 High pressure processing

HPP is a nonthermal technique to preserve food by the inactivation of pathogens and vegetative growth of microbes through pressure in place of temperature. In the HPP technique, high pressure (400−600 MPa or 58,000−87,000 psi) at $<45^{\circ}C$ is used that allows most of the food products to protect them from minor effects on flavor, texture, taste, appearance and nutrition (De Ancos et al., 2000). High-pressure processing is also demonstrated as high-pressure homogenization (HPH) and high hydrostatic pressure processing (HHP). The viability of probiotic depends upon the process parameters, that is, pressure, temperature, time and probiotic strain used in the product. The type of probiotic microorganism use in the development of functional products is very important. Probiotic should be technically suitable and compatible with the product and processing conditions so that they remain viable and efficient during processing and until consumption (Stanton, 2003). Studies revealed that the utilization of high pressure does not have any bad effects on the quality characteristics of probiotic functional food. Although sometimes it provides similar or even better sensory attributes when evaluated by the consumer. HPP technology is introduced for the production of dairy-based probiotic products; it is salient feature to investigate the milk composition (fat, protein, lactose and minerals) and

TABLE 19.1 Effect of ultrasound on probiotic in functional foods.

Dairy products	US parameters/conditions	Probiotics	Effect	References
Fermented milk	20 kHz 100 WTime 7–30 minTemperature 30°C–40°C	*Bifidobacterium* spp.	Shorten fermentation time Release of β-galactosidase Increases the production of organic acids Increases the concentration of oligosaccharide Increases lactose hydrolysis and transgalactosylation	Nguyen et al. (2009)
Yoghurt	For whey processing 20 kHz 480 WTime 8–10 minTemperature 55°C, (2304–2880 J/mL)For activation of probiotics 84–102 WTime 75–150 sTemperature 37°C (0.13 J/mL)	*Lb. acidophilus* La-5	Increase cell viability Improved organoleptic properties Activation of probiotic culture (*Lb. acidophilus* La −5) in short time	Barukčić et al. (2015)
Fermented sweet whey	For whey processing 20 kHz 480 WTime 8–10 minTemperature 55°C, (2304–2880 J/mL)For activation of probiotics 84–102 WTime 75–150 sTemperature 37°C (0.13 J/mL)	*Lb. acidophilus* La-5	Increase cell viability Improved organoleptic properties Activation of probiotic culture (*Lb. acidophilus* La −5) in short time	Barukčić et al. (2015)
Fermented milk	28 kHz pulsed United States (100 s on and 10 s off) 100 W/L for 1 h before fermentation (360 J/mL) 30 min during fermentation process (≈180 J/mL)	*Lb. paracasei*	Increase production of bioactive peptides and viable cells in the fermented skim milk as compared to untreated samples	Huang et al. (2019)

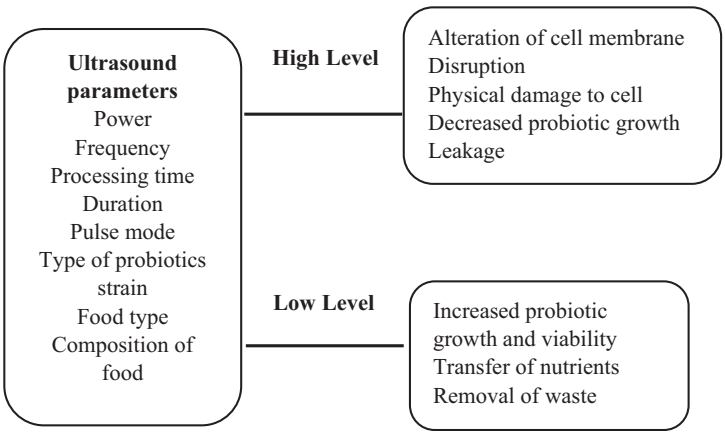

FIGURE 19.3 Effect of high intensity ultrasound on probiotic growth and cell structure.

probiotic strain profile before and after HPP treatment. The use of the HPP technique to fermented dairy products is really interesting; not only does it retain the culture alive, but also positively affects the quality parameters of the end product. Low viscosity, syneresis and low texture are the most experimental features that have been observed in yogurt that ultimately decrease its quality (Trujillo et al., 2002). Results revealed that acid-gels prepared in HPP-treated milk have improved texture (viscosity and firmness) as compared to nontreated samples. New Zealand milk processing company (Fonterra's) patented a technique to produce probiotic yogurt that has a better shelf life with high probiotic survival by selected baro-resistant strains of probiotics. HP treatment has no obstructive impact on the survival of

probiotics and keeps the viable cell count $>10^7$ CFU/mL (greater than the recommended limit). Serra and coworkers studied the proteolysis effect in the yogurt that was developed from HPP-treated milk (200−300 MPa and at 30°C−40°C) with the control. The proteolysis effect in HPP-treated milk was almost similar to conventional heat-treated milk (Serra et al., 2009). Jankowska and coworkers developed yogurt with *Bifidobacterium* spp. and *Lb. acidophilus* by using HPP processing (Jankowska et al., 2005). Results reported that both probiotic cultures managed populations of 10^7 log CFU/mL within 1-month storage at 4°C. Lanciotti and coauthors resulted that HPH treated milk improves the viability of *St. thermophiles* and *Lb. delbrueckii* spp. *bulgaricus* during refrigerated storage (Lanciotti et al., 2004).

19.4 Pulsed electric fields

In this technique, intense PEF are utilized to kill pathogenic microbes in a constant liquid medium. This electric field severity changes from 15 to 50 kV/cm, but the electric field is applied only for some seconds (Qin et al., 1998). The mechanism of action of this electric field on microbial cells is not stable due to exposure of the high-pressure pulse. It results in mechanical repute of cell, causes dents, cracks, and increases permeability and leakage of cell content (Lin et al., 2002; Wouters et al., 2001). Inactivation of microorganisms through the PEF technique depends on some factors, that is, electric field intensity, pulse duration, pulse number, microorganism profile (spore-forming or nonspore forming or vegetative growth), growth curve phase, temperature, pressure, pH, and presence of additives (Nielsen et al., 2009). As far as the application of PEF is concerned in the dairy industry, it affects casein micelles and causes coagulation of casein. It is a predominant step in the preparation of cheese. Yeom and coauthors reported that coagulation of milk is enhanced, gel firmness and time of coagulation decreases during cheese production after the application of PEF. In addition, it inactivates molds, yeast and mesophilic aerobic microorganisms (Yeom et al., 2004). There is still a need to optimize operational parameters to get better results of this nonthermal technique. Research is necessary to study the impacts of these techniques on the chemical and nutritional profile of food items along with microbiological safety and stability. There is limited research available on the effect of PEF on the endurance of probiotics in functional products. Cueva studied the effect of PEF on the growth of *Lb. acidophilus*, tolerance to gastrointestinal conditions in a pilot scale project. He reported different parameters, that is, pulse lengths (3, 6 and 9 Ms), duration of pulse (10,000, 20,000 to 30,000 Ms), electrical energy (5, 15 to 25 kV/cm) and flow speed (10, 60 and 110 mL/min). The result revealed that with pulse length applied, exposure time decreased the tolerance to bile salt and acids and also delayed the log phase of *Lb. acidophilus*. But the impact of these parameters on protease activity of probiotics is nonsignificant. On one hand, it lowers the tolerance ability to acid and bile that directly affects probiotic ability. On the other hand, it slows down the growth rate which is beneficial and results in the controlled release of bacterial enzymes that are required to develop physicochemical properties (improve taste and flavor) of products (Cueva, 2009). There is still a need to further study the effects of PEF on the survival of probiotics in products instead of studying the use PEF as a preservation technique in food. Studies should focus on chemical changes in the product composition along with probiotic survival in the products (Fig. 19.4, Table 19.2).

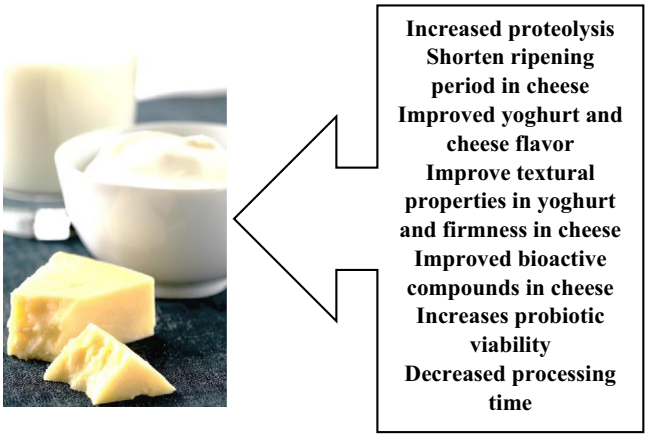

FIGURE 19.4 Effect of high intensity ultrasound on dairy products (yogurt, cheese).

TABLE 19.2 Factors affecting the viability of probiotic microorganisms.

State of damage	Stress factors	Resultant damage(s)	Selected sources
Fermentation process	Low pH values (especially less than ~5.5) Presence of food additives High redox potential High titrable acidity	Disruption through the cells, inactivation of some bacterial enzymes (starvation of cell) cell walls damage or other parts of the cellsInhibition and inactivation of cellular metabolic pathways Bacteriocidic impact after entrance into bacterial cells	Akalin and Erişir (2008), Mohammadi, Mortazavian, Khosrokhavar, and Da Cruz (2011)
Freezing process	Temperature-related stresses Chemical and biochemical stresses	Temperature decrease shock as well as the effects of frozen temperatures Condensation of detrimental solutes, dehydration of cells	Akalin and Erişir (2008), Mohammadi, Mortazavian, Khosrokhavar, and Da Cruz (2011)
Overrun process	Oxygen toxicity for anaerobic bacteria	Sensitivity of bacterial cells to metabolically produced hydrogen peroxide	Akalin and Erişir (2008), Mohammadi, Mortazavian, Khosrokhavar, and Da Cruz (2011)
Frozen storage period	Time Oxygen toxicity for anaerobic bacteria	Cells damaged during freezing die gradually during storage Sensitivity of bacterial cells to metabolically produced hydrogen peroxide	Akalin and Erişir (2008), Mohammadi, Mortazavian, Khosrokhavar, and Da Cruz (2011)
Thawing of the product	Chemical stresses	Osmotic stress, condensation of detrimental solutes	Akalin and Erişir (2008), Mohammadi, Mortazavian, Khosrokhavar, and Da Cruz (2011)

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Chapter 20

The effect of thermal processing on probiotics stability

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20.1 Introduction

Probiotics have various positive effects on human health including an immunity booster, curing some intestinal problems, improving intestinal microbial balance, treating for irritable bowel syndrome (IBS), antipathogenic, and enhancing the growth of friendly microorganisms (Antunes et al., 2020; Gu et al., 2019). To exert health benefits, the carrier products should contain 10^7 CFU/mL probiotics at the time of consumption (Shah, 2000). Mostly, the microorganisms from *Bifidobacteria* and *Lactobacillus* are included for development of different dairy and nondairy probiotics enriched food (Dias et al., 2018).

Probiotic must have genetic stability, generally recognized as safe (GRAS) status, nontoxic, adhesion ability, and resistance to hostile conditions (acid, temperature, processing, storage), Microorganisms are also required to have certain features, such as genetic stability, resistance to the gastric environment (acid and bile tolerance), adhesion capability, and good in vitro and in vivo attributes (Larsen et al., 2018).

Encapsulation is a process in which sensitive ingredients (solid or liquid) are surrounded by the polymeric matrix to ensure its stability and delivery under hostile conditions. The use of microencapsulation technology is increasing rapidly for the development of various functional foods. Immobilization or encapsulation ensures the stability of probiotics during harsh processing and storage as well as in gastrointestinal digestion conditions (Asgari et al., 2020; Chew et al., 2019; Kailasapathy et al., 2008; Martínez-Álvarez et al., 2020).

However, the results of different studies showed their poor survival and stability in carrier food (fermented and nonfermented) products (Ranadheera et al., 2017). Furthermore, their survival and stability under the gastrointestinal system are very poor due to harsh conditions. Various methods have been used in the past to prolong the viability and stability of probiotics in dairy and nondairy products. However, microencapsulation has emerged as the most effective and efficient technology to overcome the survival and stability issues of probiotics under various adverse conditions (Georgia et al., 2021; Ranadheera et al., 2017).

Encapsulation techniques are helpful in ensuring the stability and delivery of various sensitive ingredients, such as vitamins, minerals, enzymes, probiotics, pigments, antimicrobials and antioxidants. The technology is helpful in producing various capsules of different sizes. Capsules having a diameter $>5000\,\mu m$ are termed as macro while $0.2-5000\,\mu m$, and $<0.2\,\mu m$ are considered as micro and nanocapsules respectively.

Microencapsulation technology consists of different types of techniques ranging from simple to complex. Encapsulation technology makes use of various types of biomaterials having unique properties. Encapsulating materials belong to polysaccharides, lipids, and proteins. The main encapsulating materials include carrageenan, chitosan, alginate, gellan gum, and whey protein.

All wall materials provide protection to the core materials (probiotics, enzymes, antioxidants, antimicrobial, pigments, etc.) against different hostile conditions such as high or low moisture, temperature, oxidative stress, and mechanical stress (Grgić et al., 2020; Peltzer et al., 2017).

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The success of encapsulation mainly depends on the coating material, method of encapsulation, and type of microorganism (Anantal et al., 2004). The current chapter aims to give an overview of the most commonly used wall or coating materials used for the encapsulation of probiotics.

The various aspects of the wall materials have been explored through different encapsulation techniques that include: extrusion, emulsion, freeze-drying, spray dryer and liposomes. Furthermore, most commonly used wall materials mainly belong to protein, lipids and polysaccharide groups (Hamid et al., 2020; Peltzer et al., 2017).

Probiotics are live microorganisms that have a valuable effects on human health. These are the health adjuncts providing different health benefits like lowering cholesterol, preventing GI infection, improving the immune system and relieving lactose intolerance. The daily intake of probiotics should not be less than 10^8 CFU/day. Several factors are responsible for the low viability of probiotics. Factors that influence are pH, acidity, and dissolved oxygen contents (as probiotics are anaerobic), storage conditions, hydrogen peroxide produced during the fermentation of product, species, strains, the concentration of citric acid and buffer.

20.2 Stability of probiotics

The living cells of probiotics are considered to be the most important in presenting advantageous effects. Probiotic effects of these microorganisms are linked with their viability at the site of their work. For the probiotics, it is approved they should be present at 10^7 CFU/mL rate at the distribution point. The daily intake of probiotics should not be less than 10^8 CFU/mL. Viability is an important characteristic of probiotics, which is directly linked with the provision of different health benefits. Different studies have shown clinical proof of the viability of probiotics and health benefits. However, the combination of dormant and active cells can modulate the gut ecosystem depending upon the conditions. Various factors are accountable for the low viability of probiotics; these factors are pH, acidity and dissolved oxygen contents, hydrogen peroxide production during fermentation of product, species, strains, the concentration of citric acid and buffer. These factors must be examined during the production of probiotic products. To increase the viability of probiotics, we have to embrace such technology, which is efficient in giving an environment to the living cell so that their persistence in the food product is enhanced, and at the time of utilization they present in sufficient amount, which is necessary for good health of consumers. Therefore the probiotics that are used should have protective functions against the surrounding environments. The approach of protecting the viable cell is known as encapsulation (Albadran et al., 2015; Nag & Das, 2013).

20.3 Heat-processing techniques and their effect on the viability of probiotics

Heat-processing techniques are adopted to protect the food from different spoilage and pathogenic microorganisms. The application of heat inactivates the protein present within the body of the microorganism and protects the food from deterioration. The heat-processing techniques improve the digestibility and shelf life of the product. However, the use of high temperature not only affects the nutrition status of food materials but also destroys the natural beneficial microflora of the food. The probiotics present within the dairy products has been killed with the use of high-temperature processing. The probiotic-fermented dairy products have been used as a commercial source of probiotics. The probiotic starter culture used in these products is available in different forms, for example, frozen, freeze-dried culture, spray-dried or heat-processed form. The dried culture is easy to manage and transport as it has a better shelf life and covers less space. The cell viability of probiotics is distorted during drying, dehydration, and storage. The use of excessive dehydration can lead to cell damage. Similarly, the high temperature can inactivate the protein of microbes. Different processing operations in industries during the manufacturing of freeze-dried culture can lead to the inactivation of probiotic cells. The probiotic cells are freeze-dried in three different phases: freezing, primary, and secondary drying. The probiotic cells are damaged due to the freezing and drying process. Cellular inactivation crystal formation and desiccation may lead to the destabilization of cells. The cell membrane may get damaged due to the oxidation in the drying process (Khan et al., 2020; Lahtinen, 2012).

Drying is also another heat-processing technique, used to improve the shelf life and keeping qualities of the products. Probiotic strains are also dried to reduce the cost of storage and increase shelf life. The process can help to handle, transport, process, and utilize the probiotics effectively. The use of high temperatures during the drying of the probiotic product can reduce the viability of probiotics. The use of hot air, vacuum, freeze, fluidized bed, and spray drying can offer different types of stresses during processing which can reduce the efficiency of probiotics. The freeze-drying comparatively maintains the viability, but results in a costly production of probiotics. The fluidized bed drying helps to increase the air stability of probiotics during the processing of probiotics. The processing results were quite

promising, as it resulted in increased viability compared to the freeze drying process. However, the probiotics cells have to face thermal and oxidative stress during fluidized bed drying. The stressed cells have better performance in simulated gastric conditions as compare to the other cells (Feng et al., 2018; Mansouripour et al., 2013).

Spray drying is another heat-processing technique for the preparation of probiotic dried culture. The technique is relatively less expensive and suitable for the industrial scale production in a short period of time. However, cell dehydration and use of high temperature can damage the cell membrane. The probiotic cell can be inactivated due to the thermal stress. There are different types of stresses faced by the probiotic bacteria in spray drying process including oxidative, thermal, and osmotic. So these conditions may lead to the inactivation of viable cells and the resulted health benefits may not be achieved. In different small- to medium-scale production of fermented dairy products, the probiotic culture is used as freeze-dried or frozen form species (Piqué et al., 2019; Tripathi & Giri, 2014).

The large-scale producers of fermented probiotic products use the spray dried probiotic culture using controlled processing conditions. The process is six times cheaper compared to freeze drying and dehydration. The use of controlled and nonlethal temperatures can enhance the viability of different probiotics strains if managed in an effective way. The commercial use of probiotics depends upon the probiotic potential of the respective strain (Anantal et al., 2004). The probiotic strain should tolerate the harsh conditions of different stresses. The DNA and RNA of probiotic cells are damaged during the process. In spray drying, the probiotics should have to face 4 hours of heat stress, which may result in the improved survival of probiotics in gastrointestinal conditions. Albadran et al. (2015) investigated the effect of heat treatment and spray drying on *Lactobacillus* species, their viability, and resistance to simulated gastrointestinal digestion. They concluded that the use of spray drying and heat stress may induce better survival in probiotic strains during storage and consumer use. This process may help to improve the cell functionality, hence, improve the probiotic potential of the strain. However, this feature is only specific for the some of the *Lactobacillus*.

The heating of probiotics above 45°C can destroy the microbial cells. Probiotics are recommended to be present in the food at 10^7 log CFU/mL. The heating of probiotic product sat 70°C can reduce the microbial content up to 05 log CFU/mL. The tolerance of the probiotic strain plays an important role to endure thermal abuse. The pasteurization of dairy products above 60°C can result in three log reduction of probiotic cells. The addition of probiotic cells after pasteurization can improve the bacterial count at the end of the process. The thermal stability of psychrophilic and mesophilic probiotics is very low as compare to the thermophiles and thermoduric probiotics (e.g., *Bacillus* sp.). The addition of probiotic culture before pasteurization is technically desirable to improve physicochemical, textural, and sensorial attributes but not suitable. The suitability of the addition of probiotics matters with their viability. The application of high temperatures (>100°C) results in a complete loss of probiotics.

Ultra high temperature (UHT) treatment of milk destroys the natural microflora in milk. The use of probiotic culture helps to restore the recommended amount of probiotics in food. The growth and activity of probiotic strains in UHT milk is lower compared to the untreated milk. The *B. lactis* was inoculated in nutrient enriched media and incubated for 12 hour (Yerlikaya, 2014). The nonsignificant growth and activity were observed. However, the addition of mixed culture in the fermented milk can increase the activity of probiotics. Thus the UHT treated milk is not suitable for the production of fermented milk due to the lower activity and viability of probiotics (Madureira et al., 2011).

Tyndallization process is another heat-processing technique uses temperature between 70°C and 100°C to sterilize the food content. The process results in killing all microorganisms including the vegetative and spore forming. The probiotics present in different food commodities, when treated with tyndallization, are killed due to thermal abuse. The probiotics are sensitive to high temperatures. The process has lethal effects on bacterial cells, especially destroying the cell wall and basic cell constituents, rupturing the cell wall, and denaturing the protein content due to the heat stress. The pili of probiotics are important for their attachment to the epithelial lining of the gut. The use of high temperature results in the inactivation of heat sensitive pili. So the probiotic bacterial cannot attach to the lining and cannot provide the resulted health benefits (Piqué et al., 2019).

The use of high temperature techniques in the production of probiotics during the processing of probiotic and fermented product results in the loss of viability of probiotics. The heat ruptures the outer wall of the probiotics, denatures the protein content of the cell, and results in complete inactivation of probiotic cells. There are hybrid techniques like high pressure processing using the combination of heat and pressure. Tsevdou et al. (2020) evaluated the effect of different processing techniques on the viability of probiotics. They coupled high pressure with temperature to check the viability of *Bifidobacterium bifidum* and *Lactobacillus casei* in yogurt. The pressure and temperature were applied with variation. The growth kinetics were used to determine the effect on viability of the probiotics. The results suggested that using the pressure (200−300 MPa) did not have any significant effect. However, with the increase in pressure and temperature the losses in viability increased. The viability losses were calculated as 1−1.5 log CFU/g. Different techniques were used to protect the viability of probiotics including microencapsulation.

Encapsulation using probiotic is known as probiotic encapsulation technology using many microorganisms which are immobilized in globular structure of membrane and so moderates the release of cells. Sometimes a double layer is formed to protect the microorganism of interest. Biomolecules are the material used for encapsulation of the microbes. Material to be used as a biomolecule should provide maximum protection to the active material and keep the properties active during its processing and storage. There are different types of biomolecules reported in literature like polysaccharides (chitosan, xanthan, dextrin and carrageenan), milk, and milk proteins. Sodium alginate is mostly used for its low cost and easy availability (Iyer & Kailasapathy, 2005). Encapsulation effectively protects the microorganisms from the unreceptive environment and gastrointestinal tract, thus potentially preventing cell loss. The survival rate of encapsulated bacteria at pH 2.0 increased and attained a mean value of 58.9% compared with the corresponding value for nonencapsulated. Different types of coating material are used for encapsulation of probiotics, some of which are proteins (albumin, hemoglobin, and casein), celluloses (carboxy, methylcellulose), gums (sodium alginate, agar and gum arabic), lipids (wax, hardened oils and fats), and carbohydrates (starch, dextrin and sucrose) (Azam et al., 2020). Similarly, different techniques are used to encapsulate probiotics. There are many techniques for he encapsulation of the probiotics and the active ingredients. Some of these are centrifugal extrusion, stationary or immobilize extrusion, submerged or sunken nozzle, vibrating nozzle, centrifugal suspension-separation, rotating disk, spray drying, spray coating with the help of a fluidized bed, air suspension, molecular inclusion, interfacial polymerization, electrostatic deposition, pan coating, solvent evaporation, membrane emulsification and cocrystallization (Favaro-Trindade et al., 2006). Mansouripour et al. (2013) evaluated the effect of microencapsulation on the survival of probiotics during the heating process. They concluded that microencapsulation helped to protect and improve viability of probiotics (Table 20.1).

The living cells of probiotics are considered to be the most important in presenting advantageous effects for good health. Probiotic effects of these microorganisms are linked with their viability at the site of their work. For probiotics, it is approved that they should be present at 10^7 CFU/mL rate at the distribution point. The daily intake of probiotics should not be less than 10^8 CFU/mL. Viability is an important characteristic of probiotics, which is directly linked to providing different health benefits. Different studies have shown clinical proof of the viability of probiotics and health benefits. However, the combination of dormant and active cells can modulate the gut ecosystem, depending on the conditions. Various factors are accountable for the low viability of probiotics, including pH, acidity, dissolved oxygen contents (as the probiotics are anaerobic), storage conditions, hydrogen peroxide production during fermentation of product, species, strains, concentration of citric acid, and buffers. These factors must be examined during the production of probiotic products. To increase the viability of probiotics, we have to embrace such technology, which is efficient in giving an environment to the living cell so that their persistence in the food product is enhanced, and at the time of utilization they present in sufficient amount, which is necessary for good health of consumers. Therefore the probiotics

TABLE 20.1 Effect of different treatment on the probiotics stability.

Treatment	Effects	Reference
Drying	Cellular inactivation crystal formation and desiccation may lead to the destabilization of the cell. The cell membrane may damage due to the oxidation in the drying process	Lahtinen (2012)
High temperature	Use of high temperature during drying of the probiotic product can reduce the viability of probiotics	Khan et al. (2020)
Spray drying	There are different types of stresses faced by the probiotic bacteria in the spray drying process including oxidative, thermal, and osmotic. So, these conditions may lead to the inactivation of viable cells and the resulted health benefits may not be achieved	Feng et al. (2018)
Freeze drying	The freeze-drying comparative maintains the viability but results in a costly production of probiotics. The freeze-dried culture can lead to the inactivation of probiotic cells	Haddaji et al. (2015)
Pasteurization	The pasteurization of dairy products above 60°C can result in three log reduction of probiotic cells. The heat stability of psychrophilic and mesophilic is very low as compare to the thermophiles and thermoduric probiotics	Piqué et al. (2019)
Ultra high temperature (UHT)	UHT treated milk is not suitable for the production of fermented milk due to the lower activity and viability of probiotics	Moayednia et al. (2010)
Tyndallization	The probiotics present in different food commodities, when treated with tyndallization, are killed due to thermal abuse	Piqué et al. (2019)

that is used should have defenses from unfavorable surrounding environment. The approach of protecting the viable cell is known as encapsulation (Albadran et al., 2015; Nag & Das, 2013).

20.3.1 Influence of food matrix on the viability of probiotic bacteria

Evidence suggests that dietary matrices play a significant role in the positive influence of probiotics on the health. Work emphasis is on characterizing such probiotics, as well as on how the alimentary matrix and food material relates to the utmost effective probiotic. The safety of target food applications should also be taken into account, with the option of a cryoprotective product for probiotic applications. The most commercially active probiotics in the food market are *Lactobacillus* and *Bifidobacterium* species. Fermented drinks with bacteria from the probiotics are essential to human diets across the globe, as fermentation is an inexpensive technique that improves food safety, food nutrition, as well as sensory properties. Historically, the use of such probiotics in dairy products has been popular. However, provided that individuals who are sensitive to dairy proteins and/or have extreme lactose sensitivity are unable to ingest milk drinks, nondairy drinks including vegetables and fruits may also be an perfect carrier to supply customers with probiotics (Kun et al., 2008). The use of probiotic crops in multiple food matrix-based drinks may be a big challenge. There are different types of probiotics that produce various effects such as substratum acidity, postacidificated drinks, dissolved oxygen, biochemical products, and digestive conditions. Bacteria's metabolic activity and viability are essential characteristics of probiotic beverage inclusion. In order to ensure no health risks from probiotic drinks, the requirement of the least amount of viable probiotic cells ranging from 10^6 to 10^7 CFU/mL at expiration was proposed (Madureira et al., 2011).

20.3.1.1 Dairy product

Dairy products are regarded as the best-known commodity for probiotics, since the beneficial results of LABs in fermented milk have been related with the wellbeing of consumers, in addition to its own functional and nutritional features (Vasiljevic & Shah, 2008). The transition of lactose into the lactic acid reduces pH and thus facilitates milk protein precipitation. If the target pH is achieved, the gel is dissolved through the filter system and cooled directly to 20°C, which slows down fermentation, enabling the transition of yogurt to a package, and also eliminates differences throughout the gel structure. The substance is then cool down to 4°C and stored, shipped, and delivered at this temperature (Merenstein et al., 2010). Numerous lactic acid bateria (LAB) animals, including probiotics, are now used in the preparation of yogurt and the creation of new yogurts are suggested to replace dairy with functional foods. However, even though the fruit enriched fermented milks are the cause of half of the yogurt-like products industry, there are few literature publications of such fruit-enriched dairy products. One of the principal issues in the manufacture of fruit-enriched yogurt is the acidic atmosphere that most fruit will confer on the product (Kailasapathy et al., 2008).

20.3.1.2 Fruit and vegetables based beverages

The sustainability of *Bifidobacterium animalis* and *L. acidophilus* in yogurt with the addition of fruit mixtures, that is, mixed berry, mango, passion fruit, as well as strawberry, was estimated. The scientists showed that the additional fruit mixtures in various levels had no influence on the probiotics. Similar findings were reported by, who observed that as compared to the control; the counts of *L. acidophilus, S. thermophilus*, and *Bifidobacterium* spp. did not show any substantial change in fermented milks added with banana puree (Bakirci & Kavaz, 2008; Çakmakçi et al., 2012).

New and exciting methods for medicinal products include fermented fruit and vegetable juices that contain probiotics. Dairy-based beverages are of concern to individuals with lactose intolerance or milk sensitivity, so the majority of probiotic products are fruit and vegetable-based. In recent times, market demand for other than dairy probiotic products has increased considerably. Thus the focus of probiotic beverages as vegetable or fruit juices will eventually become a significant group in future. The use of probiotic crops in fruit or vegetable juices is a big task. We could assume probiotics as health benefits for customers are sufficient. However, a number of factors like oxygen levels, acidity (pH), the presence of antimicrobial substances or lack of nutrients in the product can kill these probiotics. Fruits as well as vegetables are composed of vitamins, minerals, antioxidants, and dietary fibers, which may make them the perfect substrates for the development of probiotics (Yoon et al., 2006).

The rising need for new probiotic products has encouraged the growth of nondairy foods, primarily discovering fruit juice as a standard for probiotics. Vinderola and Reinheimer (2003) detected that the regular fruit juices (kiwi, apple, pineapple, peach, and strawberry), added to development of liquid media, employed an inhibitory influence on *S. thermophilus*. The strawberry juice reserved all types of probiotic strains, excluding *L. casei*, however, the kiwi and

pineapple juices had a adverse influence on the development of *L. acidophilus* strains. Furthermore, apple juice showed the growth of *Lactococcus lactis*, while peach juice showed no consequence on any probiotic (Sheehan et al., 2007), trying the sustainability of few *Bifidobacteria* and *Lactobacilli* strains in orange, pineapple, as well as cranberry juices, detected that *L. rhamnosus, L. casei*, and *Lactobacillus paracasei* exhibited the lower-most sensitivity to the lower pH surroundings of the juices; whereas, all probiotics presented advanced counts in pineapple and orange juices as compared to the cranberry juice.

The probiotic feasibility was dependent on the strain and, generally, *L. acidophilus* was less resistant as compare to *L. rhamnosus*. The literature also recommended that storing the fruit juice up to 1 month had no substantial influence on the sensitivity of probiotics to pancreatic or bile enzymes. The pomegranate juice was effectively fermented by *L. delbrueckii* and *Lactobacillus plantarum* that were accomplished to survive during the first 2 weeks, while *L. acidophilus* and *L. paracasei* lost their sustainability (Mousavi et al., 2011). On the other hand, the effect of pomegranate tannins on the sustainability of probiotic bacteria existing in the human intestine is concerned, it was detected that *Bifidobacteria* and *Lactobacilli* were not influenced by ellagitannins. Furthermore, by adding the pomegranate by-product juice, it create considerable stimulation in the development of *Bifidobacterium infantis* and *Bifidobacterium breve*; whereas, punicalagins reserved the one of pathogenic *Staphyloccocus aureus* and *Clostridium* sp. (Bialonska et al., 2009).

20.3.1.3 Other products

In addition to fruits and fermented milks, a lot of food products have been verified as a carrier for the consumption of various probiotic strains, such as fermented acerola (*Malpighia emarginata*) ice cream, confirmed to provide probiotic bacteria sustainability. Meanwhile, the counts of *B. lactis* and *B. longum* preserved above 10^6 CFU/g throughout 15 weeks and stored even in acidic foods (pH 4.5) (Favaro-Trindade et al., 2006). The totals of *L. paracasei* and *B. lactis* persisted above 10^7 CFU/g in the coconut flan (Correa et al., 2008). Pear puree fermented by *Leuconostoc mesenteroides* was capable to keep continual probiotic counts about 10^9 CFU/g for 14 days (Kim et al., 2010). Microencapsulated *L. acidophilus* was used to ferment the banana puree, causing a symbiotic product with virtuous consumer acceptance as well as desired probiotic counts (Tsen et al., 2004). *Lactiplantibacillus plantarum* exhibited viability in malt, wheat, and barley extracts, presenting the maximum counts into the last medium (Charalampopoulos & Pandiella, 2010). Cheese is a compromised food-based product for biogenic substances and probiotic cultures, that is, bioactive peptides and conjugated linoleic acid. Associated with various other fermented products, it has a comparative fat content and high pH, a higher buffering capacity, and a solid consistency.

20.4 Conclusion

The potential health benefits associated with the consumption of particular probiotics may not be stopped owing to the substantial reduction in viability and stability during storage and gastrointestinal tract. The acid and thermal stress are the significant factors for the substantial reduction of the probiotics in carrier food. Encapsulation technology is a promising approach for the augmentation and delivery of probiotics administered orally or for the development of functional food. To increase the viability of probiotics, we have to embrace such technology, which is efficient in giving an environment to the living cell so that their persistence in the food product is enhanced, and at the time of utilization they present in sufficient amount, which is necessary for good health of consumers. Therefore the probiotics that should be used have better protective effect from unfavorable surrounding environment.

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Chapter 21

Hydrogels as carrier for the delivery of probiotics

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21.1 Introduction

The importance and marketing demand for probiotics and carrier foods is increasing across the world. The incorporation of probiotics is not only limited to fermented products. The various non-fermented products carrying probiotics are getting attention. (Nualkaekul et al., 2013). Generally, probiotics are living microorganisms which, when consumed in adequate amounts, exert beneficial effects on human health (Fareez et al., 2015). Probiotics have various positive effects on human health including immunity booster, curing of some intestinal problems, improving intestinal microbial balance, treatment for irritable bowel syndrome, anti-pathogenic, and enhance the growth of friendly microorganisms (Albertini et al., 2010). In order to exert health benefits, the carrier products should contain 10^7 CFU/mL probiotics at the time of consumption (Shah et al., 2016). Mostly, the microorganisms from *Bifidobacteria* and *Lactobacillus* are included in the development of different dairy and non-dairy probiotic-enriched food (Daly & Davis 1998; Dias et al., 2018). Probiotics must have genetic stability, GRAS status, adhesion ability, and be non-toxic and resistant to hostile conditions (acid, temperature, processing, storage). Microorganisms also need to acquire certain characteristics, such as survival in the gastric environment (acid and bile resistance), adhesion capability, genetic stability, and good in vitro and in vivo attributes (Larsen et al., 2018). However, the results of different studies showed their poor survival and stability in carrier food (fermented and non-fermented) products (Ranadheera et al., 2017). Furthermore, their survival and stability in the gastrointestinal system is very poor due to harsh conditions. Various methods have been used in the past to prolong the viability and stability of probiotics in dairy and non-dairy products. However, microencapsulation has emerged as most effective and efficient technology to overcome the survival and stability issues of probiotics under various adverse conditions (Frakolaki et al., 2021). Encapsulation is a process in which sensitive ingredients (solid or liquid) are surrounded by polymeric matrix to ensure its stability and delivery under hostile conditions. The use of microencapsulation technology is increasing rapidly in the development of various functional foods. Immobilization or encapsulations ensures the stability of probiotics during harsh processing, storage, as well as in gastrointestinal digestion conditions. (And & Kailasapathy 2005; Asgari et al., 2020; Chew et al., 2019). Encapsulation techniques are helpful in ensuring the stability and delivery of various sensitive ingredients, such as: vitamins, minerals, enzymes, probiotics, pigments, antimicrobial and antioxidants. The technology is helpful in producing various capsules with different size. Capsules having a diameter $>5000\ \mu m$ are termed as macro while $0.2-5000\ \mu m$, and $<0.2\ \mu m$ are considered as micro and nano-capsules respectively. Microencapsulation technology consists of different type of techniques ranging from simple to complex. Encapsulation technology makes use of various types of biomaterials having unique properties. Encapsulating materials belong to polysaccharides, lipids, and proteins. The main encapsulating materials include, carrageenan, chitosan, alginate, gellan gum (GG), and whey protein. All wall materials provide protection to the core materials (probiotics, enzymes, antioxidants, antimicrobial, pigments, etc.) against different hostile conditions such as high or low moisture, temperature, oxidative stress, or mechanical stress (Grgić et al., 2020; Peltzer et al., 2017). The various aspects of the wall materials have been explored through different encapsulation techniques that include: extrusion, emulsion, freeze drying, spray dryer and liposomes. Furthermore, the most frequently used wall materials are mainly belonging to protein, lipids and polysaccharides groups (Dar et al., 2020). Success of encapsulation

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mainly depends on the coating material, method of encapsulation, and type of microorganism (Dar et al., 2020). The current chapter aims to give an overview of the most generally used wall or coating constituents used for the encapsulation of probiotics.

21.2 Polysaccharides

Polysaccharides or polyglycans belong to natural polymers containing monosaccharide subunits that have hydroxyl groups; these groups might react with water or similar molecules through hydrogen bonding. Though the nature of polysaccharides is also affected by the type of their monosaccharide units and their functional groups that result in a broad spectrum of changes in their chemical and physical attributes. The functional groups can be natural polysaccharides or altered, also famous as artificially produced or partially natural polysaccharides. Irrespective of their source or conformation, polysaccharides are usually used as encapsulating ingredients for probiotics; they can be categorized into four classes based on the intensity of their charges: non-ionic, amphoteric, anionic, cationic (Gruber, 1999).

21.2.1 Anionic polysaccharides

These polysaccharides behave as negatively charged entities in a medium having a pH reading higher than their pKa value; they behave as unbiased if they are exposed to pH well below their pKa value. Gum arabic, GG, xanthan, carrageenan, pectin, and alginate are natural anionic polysaccharides commonly employed in the microencapsulation of probiotic, while usually used altered anionic polysaccharides include sodium carboxy methyl cellulose (CMC), which is commonly identified as cellulose gum, and carboxy methyl chitin (CMCH), which is also known as chitin liquid. Ionic compounds in their immediate vicinity may change the electrical potential of polysaccharides. Negatively charged compounds can react with monovalent or multivalent ions, like Na^{+} or Ca^{2+} on the organic polymer chain, altering general electrical potential attributes. The gel-forming ability of anionic polysaccharides depends upon their interference with antagonistically charged units on the polymer chain. Just like in the case of gelling properties of pectin and alginate with divalent metallic ions, such as Ca^{2+} (Matalanis et al., 2011).

21.2.1.1 Alginate

Alginate is a component extensively employed as a covering ingredient for the manufacture of probiotic microcapsules through ionic gelation in combination with spray drying and extrusion techniques. Nonetheless, as compared to alginate, pectin has proved more resistant to stomach acid and possesses similar attributes as alginates (Ribeiro et al., 2010). The source of alginate, an unbranched heteropolysaccharide, is the cell wall of *Laminaria* spp. It forms ß-1,4 glycosidic bonds among the α-L-guluronic acid and the ß-D-mannuronic acid residues in different chemical arrangements (Kwiecień & Kwiecień 2018). Contrary to this, pectin forms a surface covering structure through calcium gelification which is chiefly comprised of D-galacturonic acid subunits linked through α-(1−4) in a linear fashion. Moreover, they also form highly branched structures with some neutral sugars like xylose, galactose and arabinose (Piornos et al., 2017). Although similar in structure, alginate is still being massively used as coating material for probiotics microencapsulation.

21.2.1.2 Carrageenans

Carrageenans are composed of many sulfated polysaccharides mixtures and is isolated from Rhodophyta. Three types of chemically and structurally different commercial carrageenans (κ-, ι-, and λ-carrageenan) are produced by Rhodophyta (Chakraborty, 2017).

21.2.1.3 Xanthan gum

Many species of the family Xanthomonadaceae produce santhan gum (XG). Yet presently, XG is manufactured commercially from *Xanthomonas campestris*, which is a bacterium of plant origin. The biochemical arrangement of XG contains pentasaccharide repeating components, that are comprised of a side-chain including one glucuronic acid molecule between two mannose components, connected to each second glucose molecule of a linear molecule of cellulose (Jansson et al., 1975). The arrangement of XG also comprises different ratios of O-acetyl and pyruvyl units, which depend on the bacterial specie, and also on the fermentation conditions of bacteria (Petri, 2015). The acetyl and pyruvyl units have a key role on account of their protonation at less than 4.5 pH, which gives XG polyanionic potential attributes. The reaction between the side chains of the acetyl/pyruvyl and XG units result in crosslinking within the

molecule, increasing the changes in a foldaway structure in the XG molecule. Therefore, the XG properties are primarily affected by milieu circumstances, like the pH, behavior of the electrolyte, and the ionic strength (Petri, 2015). Recent research has revealed that the existence of acetyl or pyruvate on the external mannoses of the xanthan gum effects conform helical stability (Wu et al., 2019). Moreover, directly relating to the rheological properties of XG in liquid phase, acetyl and pyruvate content is an essential variable for its practical application. For example, higher amounts of pyruvyl components promote a gel consistency while less amounts result in less viscosity (Petri, 2015). Notably, the acetyl and pyruvyl subunits in the XG make the conditions favorable for complexation with divalent cations (e.g., Mg^{2+}or Ca^{2+}) (Bergmann et al., 2008). In many studies, XG has successfully tolerated simulated gastrointestinal conditions and high temperatures, and has stood out as a promising coating material for the probiotics microencapsulation (Fareez et al., 2017; Shu et al., 2018; Valero-Cases & Frutos 2015). Additionally, the coating characteristics of XG have been further improved by blending it with different coating substances like alginate, chitosan, gellan and β-cyclodextrin (McMaster et al., 2005; Shu et al., 2018). For example, *Lactobacillus plantarum* 12, using alginate and XG coating constituents, has successfully tolerated simulating gastric juice and bile salts and remained more viable than free cells. Therefore, the blending of chitosan to the coating complex XG-alginate augments *L. plantarum* 12 survival, providing a greater barrier against the little pH and higher temperatures (Fareez et al., 2017; Shu et al., 2018; Valero-Cases & Frutos 2015). Likewise, XG-based encapsulate materials are: the XG-chitosan-XG and XG-chitosan groups, which enhance the culture stability of *L. acidophilus* in microcapsules, used in dairy-based drinks (Fareez et al., 2017; Shu et al., 2018; Valero-Cases & Frutos 2015) and the XG-GG complex, that enhanced cell viability of microencapsulated *Bifidobacterium* lactis preserved in sodium phosphate buffer (pH 6.8) (McMaster et al., 2005).

21.2.1.4 Gellan gum

The GG is one of the anionic polysaccharides that have a linear structure composed of a tetrasaccharide-repeating sequence that comprises one α-L-rhamnose, one β-D-glucuronate and two residues of β-D-glucose. GG naturally exits in acylated form, yet its deacylation can be formed by alkaline hydrolysis. The variation in the gelation property of GG exist due to variations in acyl groups in the GG molecule. Presently, two forms of GG are available as low acyl (deacylated) and high acyl (acylated); they are also known as Kelcogel and Gelrite respectively on the commercial level. Due to the presence of gel-promoting cations, the higher acyl GG produces flexible and soft hydrogels upon cooling; whereas, the low acyl GG produces rigid and brittle hydrogels upon cooling (Zia et al., 2018). The probiotic microencapsulation has successfully used deacylated forms of GG as a coating material (Nag et al., 2011).

21.2.1.5 Gum arabic

Gum arabic or gum acacia (GA) is primarily composed of 4-O-methyl-D-glucuronic acid, D-glucuronic acid, L-arabinose, L-rhamnose, and D-galactose. The biochemical arrangement of GA is not simple. The main molecule is composed of 1−3-linked β-D-galactopyranosyl units with adjacent chains of two to five (1−6) linked β-D-glucopyranosyl units, combined with the main body by 1,6-linkages. Both main chain and adjacent branches may comprise α-L-arabinofuranosyl, β-D-galactopyranose, β-D-glucuronic acid, and α-L-rhamnopyranose units (Karlton-Senaye & Ibrahim 2013). Usually, GA is covalently linked to a protein subunit that is high in amino acid components of serine, proline, and hydroxyproline. GA in comparison to other exudates is significantly water soluble (up to 50% w/v) and also has comparatively little viscosity. The main factors involved in these characteristics are its highly branched structure and comparatively low molecular weight. Contrarily, emulsifying characteristics, foaming abilities, and surface activity of this polysaccharide are attributed to its protein fraction. Gelatin-GA (Paula et al., 2019), whey protein isolate (WPI), in combination with the specific mixture of pulp, seed, or leaf extracts of the *Synsepalum dulcificum* with the GA, were successfully utilized as coating materials for the probiotics microencapsulation compared to free cells, and significantly enhanced the stability of probiotic cells under simulated GI in vitro conditions, during processing, and during storage (Fazilah et al., 2019).

21.2.1.6 Carboxymethyl chitin and carboxymethyl cellulose

CMCH and CMC are derivatives of cellulose and chitin, respectively. They are also known as semi-synthetic anionic polysaccharides; basically modified anionic polysaccharides. Remarkably, first and second most abundant natural polysaccharides found on Earth are cellulose and chitin, respectively. The chemical arrangement of cellulose is composed of a linear arrangement of β-1,4-linked D-glucose units, whereas chitin is formed by N-acetyl-glucosamine amino sugar linked with β-1,4-linkage (Elieh-Ali-Komi & Hamblin 2016). CMCH have been extensively utilized in commercial food processing, including probiotics microencapsulation. CMCH is a water soluble derivative of cellulose. It is formed

by replacing hydroxyl groups of anhydrous glucose by carboxymethyl groups (−CH2-COOH) by reacting cellulose with alkali and chloroacetic acid (Larsen et al., 2018). A study recently proved probiotic viability during its simulated GI transit when *L. acidophilus* was microencapsulated using chitosan. In a parallel study, coating materials for probiotics microencapsulation of *L. plantarum* were produced, by blending with κ-carrageenan they revealed suitability for the production of microcapsules for oral delivery of viable probiotics (Dafe et al., 2017).

CMCH, is an anionic polysaccharide soluble in water and also known as chitin liquid. CMCH is manufactured by adding carboxy-methyl groups in place of the hydroxyl groups of chitin (Liu et al., 2016). CMCH has been extensively employed for various systems, such as cosmetic, food, antimicrobial, drug delivery systems, etc. (Narayanan et al., 2014). Yet on account of its potential, it has not been extensively exploited as coating material in the production of microencapsulation of probiotics. Recently, microencapsulation of *Bifidobacterium* was carried out by using sodium alginate and CMCH as an encapsulating material. Microencapsulated probiotics revealed a more improved viability than free cells under simulated in vitro gastrointestinal conditions, which signifies microcapsules an effective method for probiotic delivery system (Ying et al., 2016).

21.2.2 Cationic polysaccharides

The cationic polysaccharides tend to be positive under their pKa value, whereas above their pKa value, they behave as neutral. The only naturally derived cationic polysaccharide is chitosan. Chitosan is formed by partial deacetylation of chitin and is primarily made up of (1,4)-linked 2-amino-2-deoxy-β-D-glucan. Nevertheless, chitosan is also found in nature as bacterial parasites and insect exoskeletons, but it occurs in small commercially non-exploitable form. Although chitosan possesses a broad spectrum of antimicrobial potential, it is also used in combination with other encapsulating substances for probiotic microencapsulation (Sahariah & Másson 2017). Chitosan blended with xanthan gum, whey protein isolate, starch, and alginate, demonstrated an improved shield to numerous probiotics in simulated in vitro GI milieus. The stability of these microencapsulated probiotics was greatly improved against the simulated GI environment. They also showed that the probiotic microcapsules in comparison to free cells exhibited more association with the mucosal surface of fresh porcine intestinal tissues and to the EpiIntestinal™ system. Furthermore, a mouse model was used to evaluate in vivo probiotic viability, where probiotic microcapsules compared to free cells showed momentous stability(Anselmo et al., 2016).

21.2.3 Non-ionic polysaccharides

They are macromolecules with no formal charge. But, other adjacent species and/or milieu circumstances may affect their charge properties, altering their normal solution behavior. Natural, generally starch, maltodextrins, cyclodextrins, and guar gum (GUG), being non-ionic polysaccharides, have been engaged as coating materials for microencapsulation of probiotics. Moreover, their modified cellulose ethers (e.g., hydroxypropyl-and hydroxypropyl methyl- cellulose) have also been employed as coating materials (Gruber, 1999).

21.2.3.1 Starch

Starch is a tasteless soft, white powder. It is primarily produced and stored by the plants as stored energy and is composed of two fractions, amylose and amylopectin, that are formed by the repeated units of D-glucose. Amylose contains α-(1,4) linkage while amylopectin comprises of α-(1,6) linkage between the D-glucose molecules. The proportion of amylose/amylopectin in starch molecule depends upon the type of starch source and also describes its core characteristics. Strong flexible film properties are formed by the starch that might be due to principal amylose structure and its crystallization attributes; resistant starch, also known as high amylose maize starch, is a good example of this type of starch. A variety of suitable attributes exhibited by starch films make it ideal for the formulation of coating material for probiotic microencapsulation. Starch films have no taste, smell, or color. They are non-toxic and semipermeable to carbon dioxide, oxygen, moisture and also to lipids and flavor components (Nualkaekul et al., 2013). Keeping in mind these attributes of starch, for the microencapsulation of *Bifidobacterium*, an altered type of starch (octenyl-succinate starch) was evaluated as a coating material. Some starches are suitable for microencapsulation by employing the spray drying technique; the example of one such starch is octenyl-succinate, also known as E1450. This process of microencapsulation was unsuccessful compared to free cells to improve the survival of probiotic underneath acid conditions or when supplemented to dry food preparations. In spite of that, E1450 as a coating material was optimized for the manufacture of viable *Bifidobacterium* probiotic-based microcapsules through the spray drying method (O'Riordan et al.,

2001). Moreover, starch was also recommended as a prospective prebiotic compound for the microencapsulation of miscellaneous probiotics (Arslan-Tontul & Erbas 2017).

21.2.3.2 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides that catalyze the crystallization reactions of oligosaccharides and starch degrading activity; and they are produced by a bacterial enzyme that is known as cyclodextrin glycosyltransferase (CGTase). The CDs structure consists of typically six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranose units in a closed circular molecule formed by α-(1,4)-linked glucose residues (BeMiller, 2019). Presently, the most researched cyclic oligosaccharides are β-CD and their derivatives. Several benefits are linked with β-CD, like: it resists plasma cholesterol elevation; the capacity to eliminate cholesterol in various foods (e.g., dairy products and eggs); its safety and metabolism and triacylglycerols, among other benefits (Van Der Veen et al., 2000). Additionally, controlled release of drugs has been widely practiced by using cross-linked β-CD microcapsules. However, for the microencapsulation of probiotics, limited research on β-CD as a covering material have been accepted. Recently, *L. acidophilus, Saccharomyces boulardii*, and *B. bifidum* were microencapsulated by employing β-CD and gum arabic as coating materials. In a nut shell, in comparison with free cells, probiotic survivability was greatly improved in simulated GI environments and processing temperatures when encapsulated using β-CD. (Arslan-Tontul & Erbas 2017). Contrarily, (Fareez et al., 2017) reported the microencapsulation of *L. plantarum* LAB12, by using β-CD along with chitosan, alginate, and xanthan gum as coating materials. Moreover (Arslan-Tontul & Erbas 2017) reported probiotic encapsulated with β-CD exhibited a combined cholesterol lowering ability.

21.2.3.3 Guar gum

Some non-ionic cellulose like microcrystalline cellulose (MCC), hydroxypropyl methyl cellulose (HPMC), methyl cellulose (MC), hydroxyethyl cellulose (HEC), and hydroxypropyl cellulose (HPC) can also be used as coating material for the microencapsulation of probiotic. Many researchers have described the alginate blended with many other non-ionic polysaccharides as co-encapsulating material. In this regard, the studies investigated the microencapsulation of *B. lactis* 300B with the target to produce microcapsules with appropriate biochemical/physical attributes that may augment probiotic viability and the possibility to be scaled up. For the formulation of experimental coatings, the co-encapsulating components employed were MCC, sodium-carboxymethyl cellulose (Na-CMC), HPMC, two kinds of starch (BR-08 and BR-07), pullulan and dextrin. It was established that encapsulating material mixtures largely effect the probiotic survival. The two mixtures that provided maximum protection to the probiotics during 15 days of storage period and during the encapsulation process were alginate-pullulan and alginate-HPMC. Contrarily, the layer by layer method using non-ionic polysaccharides was employed the production of microcapsules of *B. lactis* in which the inner layer was grounded on HPMC while an amalgamation of HPC and poloxamer was used in the outer layer. Polaxamer is a non-ionic surfactant that is composed of a poly (ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer (PEO-PPO-PEO). Another smart polymer, comprised of HPMC/HPC/poloxamer, responds effectively to the probiotic environmental changes. Its structure shows rapid macroscopic variation dependent on a lower critical solution temperature (LCST). The polymer is soluble below the LCST, but becomes insoluble as the temperature increases above the LCST. The powdered infant formula (PIF) was added with microencapsulated probiotic, and on reconstitution of PIF the probiotic survival was tested. It was found that on reformation of PIF at a high temperature (70°C), the microcapsules protected the probiotic cells by forming an insoluble gel that protected the entrapped cells. Furthermore, the coating gel soaked when cooling at 40°C, releasing the probiotic cells (Penhasi, 2015).

GUG, also called guaran, is another natural, non-ionic polysaccharide. Seeds of the guar plant *Cyanaposis tetragonolobus* is its source. Chemically, GUG is a galactomannan that consists of a main group of D-mannose units and β-1,4-linked along with the adjacent chains of dispersed single units of α-1,6-linked D-galactose. It has been mainly designated as a coating material for drug distribution; though it was recently termed as a coating agent for probiotic microencapsulation. Recently microencapsulation of *Lactobacillus acidophilus* LA-1 was carried out (Ameeta et al., 2013) by employing fructo-oligosaccharide (FOS), partially hydrolyzed GUG, as an encapsulating mediator in alginate-starch microbeads. The survival stability of probiotics was enhanced under simulated GI milieu and during heat processing by using FOS or GUG. In another study, GUG or XG as coating materials were used for the microencapsulation of a mixed culture of three probiotic (*B. longum, L. rhamnosus*, and *L. acidophilus*). The microcapsules that were produced were then assimilated in cream cookies with the purpose to produce a functional probiotic food. Keeping this in mind, alginate and GUG as coating materials were used for the microencapsulation of *Lactobacillus* strains that were used for the addition of probiotics to milk chocolate drinks (Deshpande et al., 2019). Both studies showed that the

addition of probiotic microcapsules did not changed the taste or flavor of the final product, but still improved the probiotic viability. Contrarily, a recent study probed the use of GUG as an covering agent for the microencapsulation of probiotic yeast *S. cerevisiae*. In divergence to the preceding research, fish feed supplements were added with probiotic microcapsules. Remarkably, the use of microencapsulated probiotics were additionally shown to be favorable to the fish host, as it enhanced rates of feed conversion ratio, growth, and stimulated the immune response (Boonanuntanasarn et al., n.d).

21.2.4 Amphoteric polysaccharides

These polysaccharides carry both cationic as well as anionic charges in a single chain. The building blocks for their synthesis are polysaccharides. A few common examples are modified amphoteric starches, carboxymethyl chitosan (CMCS), N-[(2'-Hydroxy-2',3'-dicarboxy)ethyl] chitosan and, sulphated chitosan which have been used in the cosmetic industry. The amino and hydroxyl groups of chitosan are replaced with carboxymethyl groups for the synthesis of CMCS. By the modification of chitosan into CMCS some new and promising attributes are produced like ability to form good films, hydrogels, biocompatibility, reduced toxicity, more viscosity, water absorption, enhanced solubility in water (Muzzafar & Sharma 2018). Currently for the probiotics microencapsulation CMCS has been described as a coating material with good coating properties. In recent studies it was found that when microencapsulation of *Lactobacillus casei* ATCC 393 was carried out in CMCS, chitosan and alginate matrices, the microcapsules so formed enhanced the viability of *L. casei* under simulated GI conditions and cold air flow drying.

21.3 The proteins used as coating agents for probiotic microcapsules

Probiotics are the live microorganisms that have health promoting effects on humans and animals with positive effects on the physiological bioactivities of gastrointestinal micro-flora of the host. Probiotics help to improve the health of the intestine, increase body immunity, reduce the level of cholesterol and also inhibit the harmful microorganisms. However, probiotics face many challenges including poor viability, low survival rate in basic and acidic conditions. Efforts have been made to increase the efficiency and survival rate of probiotics in harsh environments and encapsulation proved very helpful in increasing the survival rate of probiotics. Various techniques are now being used to carry out the process of microencapsulation (Su et al., 2018).

Encapsulation is the process used to capture live microbes within encapsulating material; this is used as a useful tool to improve the survival of live microorganisms and delivery of bioactive molecules at the target place. Encapsulation is carried out to improve the stability of final food products and less degradation of volatile compounds. Moreover, the astringency and bitter taste of any product can also be improved with the encapsulation. Various techniques can be used to encapsulate probiotics including spray chilling, spray drying, spray cooling, liposomes, extrusion and fluidized bed coating.

Nanotechnology is the emerging technology in food sector and is unique due to novel functional properties of nanoparticles. The efficiency of microparticles and microcapsules depend on different factors, including concentration of polymers, rate of solvent removal, solubility of polymer and solubility of organic solvent in water. Different types of encapsulation material are used for different types of probiotics on the basis of plant and animal origin (Jyothi et al., 2010). The materials that are being used for the process of microencapsulation must be able to build a barrier between the internal phase and its surrounding, and be biodegradable. The most widely used materials used for the purpose of encapsulation are polysaccharides. While proteins and lipids are also being used for encapsulation. Alginate has been used for the encapsulation of probiotics because of its non-toxicity, simplicity and low cost. Gelatin is also a type of protein gum used for the purpose of microencapsulation separately or in combination with other compounds. Various other compounds including soy and pea-based isolates have also been used as encapsulating material (Dong et al., 2013).

21.3.1 Vegetable-based protein material

21.3.1.1 Soy protein

Soy protein is a type of protein that is isolated from the soybean. It is formulated with the soybean meal, which is defatted and dehulled prior to use for humans. These defatted soybean meals are then processed into three forms including soy flour, soy protein concentrates and soy protein isolates. Isolates of soy proteins have been used in the pharmaceutical and medical industry because of their functional properties. Soy protein isolates are not used for the

encapsulation of heat sensitive materials including live microorganisms. The delivery system of probiotics, based on the use of microbial transglutaminase and soy protein isolates, is reported with having increased numbers of probiotics delivered. Probiotics when encapsulated with the soy protein isolates did not show degradation of structure while passing through the harsh conditions of the intestine (Yew et al., 2011). The results of various studies showed that soy protein isolates having agro-wastes are proven to be the useful transporters for the probiotics while passing through the stomach to small intestine. The encapsulation methods preferably used for the soy protein include spray drying and freeze drying (González et al., 2016).

21.3.1.2 Pea protein

One type of food is pea protein and is a source of protein. It is extracted from split peas which belong to the group of legumes. This crop is used as a supplement to increase the protein intake of protein-deficient individuals and also is used as the substitution in various other food products. Peas are very important part of human diet due to their high protein content. Pea protein isolates, along with the alginate matrix, were used for the encapsulation of *B. adolescentis* and FOSs through the method of extrusion. The method of microencapsulation used for pea protein is spray drying. The literature proved that plant-based materials can be used for encapsulation of probiotics, and this can also improve the release rate of the respective probiotic. The survival of encapsulated probiotics is increased when traveling through the acidic conditions of the stomach (Piornos et al., 2017).

21.3.1.3 Alginate-based material

Alginic acid or algin is a polysaccharide present in cell wall of brown algae. It forms gum when hydration is performed. The salts of algin are called alginates; it forms salts with sodium and calcium, which later are called sodium alginate and calcium alginate, respectively. Alginate has two structural units including D-mannuronic acid and L-guluronic acid. It is a useful matrix for the immobilization of cells. It is also very beneficial for the entrapment of the probiotics and is delivered at the target place in a controlled system of probiotic release. The encapsulation technique used for alginate-based materials is ionic gelation. Alginate can also be used separately or in combination with other materials to protect the probiotics and to increase the viability of probiotics along with its role in increasing the survival rate of microencapsulated probiotics. Salts of alginate are also being used in the pharmaceutical industry as well as the food industry for improvement of the survival rate of nutrients and for their safe delivery to the target place. The nutrients are also protected from the acidic environment of the stomach so that the safe delivery of probiotic to the target site can be made (Piornos et al., 2017).

21.3.1.4 Cereal protein

Any type of grass which is cultivated for its edible portion, including endosperm, bran and germ, are included in the group of cereals. Cereal crops are the most cultivated crops throughout the world and are used to provide energy to the growing population. Cereals are also a rich source of vitamins, minerals, oils, fats and protein. The nanoparticles of cereal protein are being used to encapsulate, deliver and protect a variety of bioactive agents. The nanoparticles derived from cereals play an important role in preventing the bioactive components from degrading in food, and further increase the bioavailability of these bioactive components in the gastrointestinal tract. A study conducted on microencapsulation and the bio-accessibility of curcumin was enhanced when encapsulated with a cereal-based protein including zein (derived from corn), gliadin (derived from wheat) and kafirin (derived from sorghum). The stability, bio-accessibility and antioxidant capacity of quercetin have also been improved by encapsulating quercetin with gliadin and zein nanoparticles. The double emulsification and heat polymerization method was used for the encapsulation of wheat-based products while spray drying was used for barley-based products (Zou et al., 2019).

21.3.2 Animal-based protein material

21.3.2.1 Gelatin

Gelatin is a colorless food ingredient which is derived from collagen originating from the body parts of animals. After hydrolysis of gelatin, it is converted into gelatin hydrolysate, hydrolyzed gelatin, collagen hydrolysate and various other compounds. Usually it is used as the gelling agent in food, medication, vitamin capsules, cosmetics, paper and photographic films. Gelatin is also used for the encapsulation of probiotics alone or in combination with other materials. Various techniques have been used for the microencapsulation of gelatin-based products. Gelatin microspheres were

prepared and coated with the alginate and were used for the microencapsulation of probiotic *B. adolescentis*. Gelatin microspheres were cross-linked with the genipin while the alginate microsphere were cross-linked with the calcium ions. Alginate prevents gelatin from the degradation that the gastric enzymes can cause and as a result of this, the survival rate of probiotics has been increased to the significant extent. After passing through the acidic and basic conditions, the count of probiotics was improved significantly for the gelatin microsphere with alginate coating while the count of uncoated gelatin microspheres was very low when compared with the microencapsulated probiotic count (Dong et al., 2013).

21.3.2.2 Dairy proteins

Due to their suitable functional properties, dairy proteins are also being used for the purpose of encapsulation. Milk proteins can easily be encapsulated into any type of hydrophobic or hydrophilic and to viable probiotic cells. The dairy proteins are known to increase the resistance of probiotics against the acidic and environmental conditions that is why dairy protein-based microbeads can be used as the vehicle for probiotics. Various types of dairy proteins are being used for the purpose of microencapsulation, while the most important among all materials include whey protein and casein protein. The survival and release rate of the probiotics was also increased when dairy-based protein was used for the purpose of encapsulation. Whey protein is extensively used as encapsulating material for probiotics and is degraded prior to the use for microencapsulation. The degraded whey protein is proved to increase the stability of probiotics and it also helps in the survival of probiotics in acidic and environmental conditions of the gastrointestinal tract (Gerez et al., 2012). Several products of whey proteins have been used for encapsulation ranging from whey powder (15% protein) to whey protein isolates (90% protein). Studies have proved that the nature of whey protein plays a very important role in the entrapment of probiotics. Various techniques have been used for the encapsulation of probiotics with whey protein isolates; some of those techniques include extrusion, emulsification, spray drying and coacervation.

Formation of gel at room temperature by enzymes and acids is the characteristic of casein and caseinates. Casein provide the suitable conditions for the microencapsulation of probiotics and the viability of the probiotics is not disturbed. Casein is used for microencapsulation in various different forms including native casein and sodium caseinate. Capsules of casein provide protection to probiotics at low pH conditions. When casein is used with the degraded whey protein isolates, it results in the formation of average size beads along with slow changes in the shape of beads during digestion. This resulted in the best survival of probiotics (99%) with encapsulation rate (97%); these results are much better than the results found for casein and whey protein isolates separately.

Use of sodium caseinate as an encapsulation material results in the reduction in fermentation time and enhances the consistency and appearance of probiotic-based yogurt. Sodium caseinate helps the bacterial cells to pass through the acidic conditions of the stomach and to increase the viability and stability of probiotics. The examination of microencapsulated probiotics showed that the high viability of bacterial cells was retained at the storage temperature of 25°C with the relative humidity at 50%. The microscopic examination of microencapsulated probiotics showed that bacterial cells remain entrapped in the capsules while passing through the acidic conditions of the stomach and are released from the capsule when it reaches the intestinal fluid. Literature showed that the viability of probiotics becomes 100% when sodium caseinate was used as encapsulating material for various probiotics (Abd El-Salam & El-Shibiny 2015).

21.3.2.3 Egg white

Different types of globular proteins are present in egg white including ovalbumin, ovomucin, ocotransferrin and ovomucin. The temperature for denaturation of egg white protein is significantly low compared to whey and soy protein. This low degradation temperature is an important characteristic for its application in the food industry. Egg white protein can also be used as an emulsifier to stabilize the oil in water. Microgels loaded with egg white protein can be used as the delivery system for the encapsulation of lipophilic bioactive agents. Alginate is a type of plant-based protein material used for the encapsulation, protection, retention and release of probiotic molecules. Heat treatment of the egg white proteins for microgels purpose increased encapsulation and retention capacity. Protein loaded microgels are effective for food applications. When egg white was used as encapsulating material along with alginate, it resulted in the improvement of pH stability and survival of probiotics in the gastric environment. The encapsulation and retention time of egg white protein is enhanced with the thermal treatment prior to the microgel formation; this will result in the increase of physical dimensions because of aggregation and unfolding. Egg white is the richest source of protein and can also be used as the encapsulating material for the protection of bioactive components (Shu et al., 2018).

21.4 Future trends

Microencapsulation is an emerging technology used for the safe delivery of bacterial agents in the intestine, as the extremely acidic conditions of the stomach can damage the bacterial agents. The materials used for the purpose of microencapsulation prevent the probiotics and ensure the safe delivery of these bacterial agents as well as various nutrients of food to the specific target site. The materials most used for microencapsulation are carbohydrate based, but protein-based materials are equally important for the safe delivery of probiotics. This protein can be plant or animal-based and both types are equally effective in microencapsulation depending on the environmental conditions and method of encapsulation. Microencapsulation is the useful process for the safe delivery of probiotics and thus should be promoted to get more benefits from the gut-friendly bacterial community.

21.5 Lipids as edible coating materials for encapsulation of probiotics

Lipids belongs to a miscellaneous set of molecules that include fatty acids, phospholipids, fats, waxes and others. Lipids are also used as ingredients of an edible coating due to the fact that they primarily inhibit the transport of moisture due to low polarity. On the other hand, lipids hydrophobic nature confers crumbliness to the formed coatings. For this purpose, lipids are amalgamated with other coating materials such as polysaccharides or proteins, with the intention to improve their coating characteristics (Aydin et al., 2017). Some substances such as proteins and polysaccharides allow selective permeability to gases (O_2 or CO_2) along with stability, consistency, and reliability; however, when lipid is added in the mixture it improves its water vapor resistance.

21.5.1 Fats

Mostly lipids that are employed for the microencapsulation of probiotics belong to a group of fats. The source of these edible fats is either a plant group or animal origin. Some examples from plant-based fats are olive, sunflower, and corn oils. Animal fat examples are butter, fish and pork oils. All these fats naturally occur as mono-, di- or tri-glycerides and are composed of glycerol and fatty acids. Hence, most of the characteristics and properties depend on the composition of fatty acids. These fatty acids are hydrophilic carboxylate groups that are linked covalently to the other hydrophobic end with a different number of hydrogen and carbon atoms that describe the molecular weight of that fatty acid. The molecular weight of a fatty acid is linked with its melting point; the greater the molecular chain, the greater the number of carbon atoms, and the higher the melting point. Another factor that depends on the melting point is the unsaturation of the hydrophobic carbon chain. The fatty acids that have a higher degree of saturation have a higher melting point than unsaturated fatty acids. Fat's melting point is the chief property that is examined for microencapsulation, as thermal solidification prompted at temperatures that is below the melting point of fats (Aydin et al., 2017). The fats that are produced from vegetables are widely used as an ingredient in probiotic microencapsulation either by emulsion method, or by spray drying method (Zou et al., 2019). Contrary, in another study by Silva et al., it was reported that vegetable fat and gelatin-gum arabic can be used as a main encapsulating material of probiotic microencapsulation. The probiotic beads remain protected in adverse environmental simulated gastric conditions using these types of coating materials in comparison to the free probiotics without any coating material (Silva et al., 2018). Although the survivability of the microencapsulated beads still needs more improvement even during the storage period.

21.5.2 Waxes

One of the commonly used lipids in the food industry includes waxes. The naturally occurring waxes include carnauba wax, beeswax and candelilla wax; however, the examples of synthetic waxes includes oxidized polyethylene wax and paraffin. The waxes that occur naturally are the long complex organic molecules chains of ketones, aldehydes, fatty acid, esters, alcohols, fatty acids and alkyl groups and they might contain aromatic compounds. The most common example of natural wax is the honeybee wax that is the secretion of honeybees for their comb structure construction. Commonly, it is composed of saturated hydrocarbons (C25−C33), higher monohydric alcohols (C24−C33) and long chain of fatty acids C24−C34. The melting point of waxes is in the range of 61°C − 65°C; however, it has a good solubility in other oils and waxes as well. Honeybee wax is solid (plastic) at ambient temperatures; however, at lower temperatures, it shows properties like a fragile material. Candelilla wax has a melting point of 65°C − 68.8°C and is a natural secretion of candelilla plant (*Euphorbia cerifera*, E. *antisphylitica*). Its structure is composed of higher molecular weight esters, hydrocarbons (C29−C33), resins and free fatty acids. Its thickness is

between carnauba wax and beeswax. While carnauba wax is obtained naturally from the leaves of the Tree of Life (*Copernica cerifera*), however, its MP is 82.5°C − 86°C. It mostly consists of saturated long-chain alcohols and saturated fatty acid esters (C24−C32). Carnauba wax has the highest specific gravity and highest melting point than other natural waxes. Because of this reason, this wax is used to increase the stiffness the luster of lipidic blends, melting point and strength. Alternatively, the synthetic waxes mostly used in the food industry are oxidized polyethylene wax and paraffin wax. They both are obtained from petroleum-like products and shaped into powder form, pallets form, flakes form, etc. Paraffin wax is also a well-known food additive (E914) composed of hydrocarbons having generic formula CnH2n + 2, and s carbon chain length of C18−C32. Oxidized polyethylene wax has a melting point of 97°C − 115°C. It is a food additive with a code E914. However, it can be defined as one of the mild oxidation products of the polar reaction of polyethylene (Hall, 2011). The above mentioned waxes are considered as generally recognized as safe (GRAS) and are used widely in the food industry as protective coatings for cheese, fruits and vegetables, and as food additive; however, waxes are hardly employed as an encapsulating material for the microencapsulation of probiotics. In a study by (Mandal & Hati, 2016), stearic acid or poly-L-lysine and beeswax were used as an encapsulating material for probiotic microencapsulation along with alginate and resistant starch. The microbeads prepared with stearic acid and beeswax exhibited better survivability of *L. casei* in in vitro study. However, the coating of stearic acid displayed better protection and exhibited the complete discharge of encapsulated probiotics in simulated colonic pH solutions. In another study by Paula et al. (2019), the use of stearic acid or beeswax is described as an external coating of probiotic microbeads that are prepared along with cellulose-acetate-phthalate (CAP). Although, it was suggested that microbeads coated with beeswax revealed the maximum survival of *Bifidobacterium pseudolongum* even in simulated gastrointestinal conditions.

21.5.3 Phospholipids

Another class of lipids used for encapsulation in food industry are the phospholipids. Phospholipids have the capability to form emulsions, liposomes and micelles. This group of lipids have phosphorus and play a vital role in the structure and metabolism of a living cell. These lipids are more complex than other classes of lipids (fats and waxes). Some common phospholipids found include phosphatidylethanolamine (cephalin) (PE), phosphatidylserine (PS), phosphatidic acid (phosphatidate) (PA) and phosphatidylcholine (lecithin) (PC). The basic structure of phospholipids have a phosphate group that is esterified to the molecule of glycerol which may have one or more esterified fatty acids. It may also be esterified to an alcohol group and provide charge to the hydrophilic molecule. The tail of the fatty acid delivers a neutral net charge and offers hydrophobic properties to the molecule. All these properties provide the amphipathic property to the phospholipids which is crucial for the development of biological membranes (Zou et al., 2019). Hence, all phospholipids are the basic constituents of liposomes. When dispersed in the water, these phospholipids molecules form a combined characteristic bilayer and it is a significance of the collaboration between the hydrophilic water environment and the hydrophobic fatty acid chains. These interactions endorse the development of sealed and closed vesicles that are the liposomes. This liposome formation is an alternative technique that is now widely used in the food industry (Jeyakumari et al., 2016). They are extensively used as an important distribution system of bioactive compounds, i.e., vitamins, drugs, enzymes, etc. Although liposomes have displayed an important potential for encapsulation and controlled release of nutritional compounds, their application in foods has yet to be fully explored. Limited research on microencapsulation of probiotics by liposome entrapment is present, which may be because of the cost related to the process and the materials, along with the large size of probiotic microorganisms (Sarao & Arora 2017).

21.6 Conclusion and future remarks

Probiotics are getting attention across the global market because of their potential in health improvement as well as in treatment of various gastrointestinal and non-gastrointestinal diseases. The core objective of this chapter was to explore the potential applications of various coating or wall materials for encapsulation to enhance the survival and stability of probiotics under hostile conditions. Most of the coating materials have good protection properties to prolong the viability of probiotics. It can be concluded from the cited literature that most polysaccharides and protein-based materials have the ability to withstand various environmental as well as hostile GIT conditions. Furthermore, encapsulation technology is a useful technology to enhance the survival and stability of probiotics under stressed conditions.

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Chapter 22

Dairy-derived antimicrobial substances: microorganisms, applications and recent trends

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22.1 Introduction

The amino acid chains that are inactive in the structural protein, but have important physiological roles with their specific properties when released due to enzymatic activity, are defined as "bioactive peptides" (Froetschel, 1996). Bioactive peptides can be obtained from sources such as fish (Buonocore et al., 2019), plants (Chai et al., 2019), dry sausages (Gallego et al., 2018), or beef (Kęska et al., 2019), however their main source is milk and dairy products. Therefore, dairy-derived bioactive peptides are a topic that the food industry has focused on in recent years. Antimicrobial peptides (AMPs) constitute the main bioactive peptide group; the others are stimulant-opioid, antihypertensive, antithrombotic peptides.

AMPs could be obtained from several proteins such as casein, α-lactalbumin, β-lactoglobulin, and immunoglobulin in milk or dairy products enzymatically or microbiologically. There are a lot of studies on the production of AMPs, but most researchers focused on AMPs that are produced by lactic acid bacteria (LAB). The organic acids are formed microbiologically during the ripening of dairy products, mostly from different types of cheeses.

Although the mechanism of action of AMPs varies, depending on the type of bacteria and environmental conditions, they generally ensure to inhibit microorganisms by damaging either the cell membrane or the cytoplasmic membrane. The mechanism of action of organic acids depends only on increasing acidity.

In this review, the production, action mechanisms, and the health effects of bioactive peptides and other milk-derived antimicrobial substances synthesized mainly by LAB are discussed.

22.2 Dairy-derived bioactive peptides

Depending on the proteolytic activity, peptides with different properties are formed. Among these, bioactive peptides are critical in terms of their functional properties. While bioactive peptides cannot show activity in the protein sequence, they become active when they are hydrolyzed by digestive enzymes in the human body or by the enzymes secreted from microorganisms (Otağ & Hayta 2013). Dairy products are known as the primary source of bioactive peptides.

Bioactive peptides are basically divided into four main groups according to their functional properties. These groups consist of the stimulant-opioid, antihypertensive, antithrombotic, and AMPs. Apart from these properties, peptides have also known with their antioxidant (Samaranayaka & Li-Chan, 2011), anticarcinogenic, hypocholesterolemic, and mineral binding properties (Tagliazucchi et al., 2019).

22.2.1 Stimulant-opioid peptides

Bioactive peptides can have a stimulating or opioid effects on the nervous system. Unlike stimulant peptides such as casokine and lactoferoxin, opioid peptides have a relaxing impact on the nervous system (Gobbetti et al., 2002). The most known peptides with opioid properties are exorphins and casomorphins (Şanlıdere & Öner 2006). Among these

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peptides, β-casomorphine has the strongest effect (Schanbacher et al., 1998), in terms of supporting pain reduction, prevention of diarrhea, and insulin secretion (Şanlıdere & Öner 2006).

22.2.2 Antihypertensive peptides

Antihypertensive peptides are also known as angiotensin-converting enzyme (ACE) inhibitory peptides. Angiotensin is a hormone that functions as a vasoconstrictor and increases arterial blood pressure (Park, 2009). Angiotensin I-converting enzymes change angiotensin I to angiotensin II and thus increase blood pressure. ACE-inhibiting peptides prevent this transformation and regulate blood pressure (Park & Nam 2015). Studies have shown that casokines and lactokines obtained from casein and serum proteins, respectively, are accepted as ACE-inhibiting and antihypertensive peptides (Daliri et al., 2018). This effect may differ depending on the change of milk source.

22.2.3 Antithrombotic peptides

These peptides reduce or prevent blood clot formation. Caseinomacropeptide, which are formed due to the breakdown of κ-casein by renin enzyme, bind to thrombocyte receptors to avoid blood clotting or coagulation of the formed clots.

22.2.4 Antimicrobial peptides

AMPs could be defined as low molecular weight compounds composed of amino acids that exhibit a wide range of antimicrobial activity against gram-positive and gram-negative bacteria, fungi, and viruses (Izadpanah & Gallo 2005).

The antimicrobial activities of peptides occur by damaging the cell membrane of microorganisms or the permeability of the cell membrane (Akalın 2014). Peptides with antimicrobial activity include α-lactalbumin from milk serum proteins, β-lactoglobulin and casein derivatives, κ-casein, β-casein, αs_1, and αs_2-casein (Akalın, 2014). Many studies have focused on the antimicrobial activity of peptides obtained from dairy products; the antimicrobial effect of αs_2-casein derived from cow milk (Liu & Pischetsrieder 2018), DMPIQAFLLY peptide isolated from fermented cream (McNair et al., 2018), or the Casein201 peptide isolated from human milk (Zhang et al., 2017) has been proved.

There is a subgroup of AMPs specifically named "bacteriocin." Bacteriocins are synthesized by ribosomes produced by virtually cellular organisms. They differ from AMPs in some of their properties. AMPs can damage all types of microorganisms, including bacteria, yeasts, molds, and viruses, while bacteriocins had an adverse effect only on bacteria, particularly for gram-positive bacteria. The most known bacteriocins are lactococcin produced by lactococci, nisin, lactostrepsin, diplocoxin, acidophulicin produced by lactobacilli, acidoline, plantaricin, enterocin produced by enterococci, pediocin produced by pediococci, and leucosine produced by *Leuconostoc* (Üstündağ & Yalçın 2017).

22.2.4.1 Health effects of antimicrobial peptides

The main health effect of AMPs derived from milk is their protective effect against many gram-positive and gram-negative pathogens, including *Escherichia* (*E.*) *coli*, *Salmonella* (*Sal.*) *typhi*, *Bacillus* (*B.*) *cereus, Sal. typhimurium*, *Staphylococcus* (*S.*) *aureus* that adversely affects human health (Mohanty et al., 2014). Thus, AMPs act as a line of defense that can be developed in inflammation or trauma. Also, AMPs isolated from milk are AMPs that not only provide a healthy diet but also alleviate the consequences of a lifestyle disease (Mohanty et al., 2014).

Microorganisms can get into the human body in different ways. One of them is infections through the skin. In fact, intact skin provides a very significant physical barrier against microbes; therefore, skin infections rarely occur in healthy intact skin. However, wounds or damage causes cracks in the physical barrier of skin which increases the possibility of infection. In successful wound healing, it is necessary to keep the wound away from infection. Generally, chronic skin wounds are most commonly infected with *S. aureus* or *Pseudomonas aeruginosa* (Edwards & Harding 2004). AMPs play an important role in antimicrobial defense during wound healing (Sorensen 2016). Among AMPs, lactoferrin and lysozyme have been reported with increased epidermal expression during the proliferative phase of wound healing (Roupé et al., 2010).

Dairy-derived AMPs also were determined with positive oral and dental health effects (Nongonierma & FitzGerald 2015). A study with humans wearing dental appliances showed that caseinophosphopeptide-amorphous calcium phosphate could increase pH through its buffering capacity and reduces enamel mineral loss (Caruana et al., 2009). Another study showed that caseinophosphopeptide-amorphous calcium phosphate fluoride reduced bacterial growth in white spot lesions (Beerens et al., 2010).

Furthermore, AMPs play an important role in the immune response, as they exert immunomodulatory, cytotoxic, and other activities apart from direct antimicrobial effect (Diamond et al., 2009; Konovalova et al., 2018). Antimicrobial and

TABLE 22.1 Enzymatically obtained antimicrobial peptides.

Peptide (source)	Enzyme	Pathogens	References
Casecidin	Chymosin	*Staphylococci, Sarcina, Bacillus (B.) subtilis, Diplococcus pneumoniae* and *Streptococcus pyogenes*	Lahov and Regelson (1996)
Casecidin-like antibacterial peptide (camel milk β-casein)	Pepsin	*Escherichia coli* ATCC 25922	Almi-Sebbane et al. (2018)
Isracidin (αs-1-casein)	Chymosin	*Staphylococcus aureus, Candida albicans*	Lahov and Regelson (1996)
Casocidin-I (αs-2-casein)	Trypsin, pronase or endoproteinase	*E. coli* and *Staphylococcus carnosus*	Zucht et al. (1995)
residues (αs-1-casein)	Pepsin	*B. subtilis, Listeria innocua, Sal. typhimurium*, and *Sal. enteridis*	McCann et al. (2006)
k-casein (Human milk)	Pepsin	*E. coli, Staphylococcus carnosus* BL 21	Liepke et al. (2001)
Kappacin-(bovine caseinomacropeptide)	Chymosin	*Streptococcus mutans, E. coli*, and *Porphyromonas gingivalis*	Malkoski et al. (2001)
κ-casecidin	Trypsin	*S. aureus, E. coli*, and *Sal. typhimurium*	Matin et al. (2000)

immunomodulatory activities are known to be interrelated. For example, peptides derived from milk with immunomodulatory effects have been shown to increase resistance against pathogens in the gastrointestinal system (Gauthier et al., 2006).

22.2.4.2 Production of antimicrobial peptides

Milk proteins are the primary source of bioactive peptides and hence AMPs. Although AMPs are obtained from both serum proteins and casein, most of these peptides are derived from the degradation of casein. Therefore, each peptide group is explained separately below:

Enzymatically obtained antimicrobial peptides

Casein or whey-derived peptides can be obtained due to the activity of enzymes, especially chymosin and pepsin. AMPs are frequently obtained from different casein types in many different ways (Exposito et al., 2006). The enzymes used for the production of AMPs and the microorganisms affected by those peptides are given in Table 22.1.

Microbiologically obtained antimicrobial peptides

Apart from the enzymatically obtained peptides mentioned before, AMPs can also be obtained microbiologically. During enzymatic extraction of peptides, the protein is degraded, while in microbiologically obtained peptides a new peptide with antimicrobial property is formed. This is usually called bacteriocins which are particularly derived from LAB. These peptides are given in Table 22.2.

LAB are the main bacteria capable of producing bacteriocin. *Lactobacillus* (*Lb.*), *Streptococcus* (*Str.*), or *Bifidobacterium* (*Bf.*) are the most preferred LAB species for this purpose. The bacteriocin production of *Str. thermophilus* is dated back to ancient times (Mindich, 1966). Bacteriocins obtained from *Str. thermophilus* have taken different names such as Thermophilin 13, Thermophilin A, and Thermophilin 110 Gul et al. (2012). Barefoot and Klaenhammer (1984) studied the production of bacteriocins by *Lb. acidophilus* and they called this bacteriocin "Lactacin B." Many different bacteriocins produced by *Lb. acidophilus* like lactacin F, Acidocin A, Acidocin B, Acidocin JI229 and Acidocin 1B were determined later (Ahmed et al., 2010). Moreover, it was reported that *Bifidobacterium* species also produces bacteriocin. Bacteriocins produced by *Bf. bifidum, Bf. longum*, and *Bf. lactis* are called as Bifidin (Bifidocin B), Bifilong and Bifilact Bb-12, respectively. (Martinez et al., 2013). Other than the mentioned LAB, *Lb. salivarius* (Messaoudi et al., 2013), *E. faecalis/E. faecium* (Nes et al., 2007), and *Lb. plantarum* (Sabo et al., 2014) also have the ability to produce bacteriocins.

TABLE 22.2 Microbiologically obtained antimicrobial peptides.

Peptide	Lactic acid bacteria	Pathogens	References
Neutralized cell free supernatants	*Enterococcus* (*Ec.*) *faecium*	*Listeria monocytogenes* ATCC	Bagci et al. (2019)
Casecidins A and B	*Lb. acidophilus* DPC6026	*Enterobacter* (*Eb.*) *sakazakii*, *Cronobacter muytjensii*, *Staphylococcus aureus*, and *Sal. enterica* serovar *typhimurium*	Hayes et al. (2006)
DMPIQAFLLY	*Lb.* cultures	*Debaryomyces hansenii*	McNair et al. (2018)
Bacteriocin	*Streptomyces bottropensis*, *Streptomyces nigrescens*, *Streptomyces avermitilis*, *Streptomyces griseus*, *Streptomyces violaceoruber*, *Streptomyces hygroscopicus*, *Streptomyces pristinoespiralis*, and *Streptomyces rochei*	*Micrococcus luteus*, *Bacillus aerius*, *Salmonella* spp., *Bacillus wiedmannii*, *Str. canis*, *Ec. casseliflavus*, *Bacillus cereus* strain JCM 2152, *B. cereus strain* 183, *Listeria monocytogenes*, *Listeria innocua*, and *Vibrio parahaemolyticus*	Hernández-Saldaña et al. (2020)
Bacteriocin	*Streptococcus thermophilus* SBT1277	*Clostridium butylicum*, *Clostridium sprogenes*, *Bacillus cereus*	Kabuki et al. (2007)
Bacteriocin	*Lb. plantarum* zrx03	*Escherichia coli* ATCC 67387, *S. aureus* ATCC 25923, and *Listeria monocytogenes* CICC 21633	Lei et al. (2020)
AMP	*Lb. rhamnosus* C6	*E. coli* ATCC 25922	Rana et al. (2018)
Bifidin I	*Bf. infantis* BCRC	*Listeria monocytogenes*	Cheikhyoussef et al. (2010)
Salivaricin	*Lb. salivarius* UCC118	*Listeria innocua* DPC3572 and *L. monocytogenes* NCTC	O'Shea et al. (2011)
Bacteriocin	*Lb. brevis*	*S. aureus* NCTC-7447, *E. coli* NCTC-10418 and *Sal. typhi*	Rushdy and Gomaa (2013)
Bacteriocin	*Lb. plantarum*	*S. aureus* ATCC 29213, *E. coli* ATCC 25922, *Sal. typhimurium* 14028, *Sal. choleraesuis* ssp. *choleraesuis* serovar choleraesuis 10708, and *Listeria innocua* 33090)	Aguilar-Toalá et al. (2017)

Novel techniques for production of antimicrobial peptides

Depending on the technological developments, the AMPs could be produced by newer techniques. The peptide synthesis methodology of recent studies also differs from previous studies in terms of the production method.

The chromatography-based techniques such as ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography are considered traditional methods (De Vuyst & Leroy 2007), while expanded bed adsorption, a macroporous monolith, two-phase aqueous extraction, and aqueous micellar two-phase systems which have potential benefits such as high yield, low cost, shorter time necessity, are shown as a novel (Jamaluddin et al., 2018). However, since they are newer compared to the enzymatic and microbiologically extraction methods mentioned in the previous section, chromatographic techniques are also examined in the novel techniques (Table 22.3).

22.2.4.3 Action mechanism of antimicrobial peptides

There may be different mechanisms of action for each AMP. However, these mechanisms are generally based on similar structures. All AMPs interact with the bacterial membrane and tend to be divided into two classes based on their mechanism: non-

TABLE 22.3 Antimicrobial peptides obtained by novel methods.

Peptide	Production technique	Pathogens	References
CAMP211–225	Science Peptide Biological Technology	*Escherichia coli* ATCC 25922, *Yersinia* (Y.) *enterocolitica* ACTT 23715	Wang et al. (2020)
Casein201	The solid-phase method	*Staphylococcus aureus* and *Y. enterocolitica*	Zhang et al. (2017)
αs_2-casein	UHPLC–ESI–Ms/MS	*Bacillus subtilis*	Liu and Pischetsrieder (2018)
FW-2, FW-3, FK-4, and FR-5	Solid-phase method	*E. coli* ATCC 25922 and *S. aureus* ATCC 25923	Gu et al. (2020)
Lactoferrampin	Peptide synthesizer	*Candida albicans*	van der Kraan et al. (2005)

membrane destructive and membrane destructive. The outer membrane of bacteria creates a semi-permeable barrier against various substances. The outer membrane bilayer consists of an inner monolayer containing phospholipids and an outer monolayer composed mainly of lipopolysaccharides. Lipopolysaccharides localized in the outer membrane are the first target of all antimicrobial substances, especially gram-negative bacteria (Lizzi et al., 2009). The monolayer of lipopolysaccharides is a semi-crystalline structure that prevents the rapid diffusion of hydrophobic solutes. Since lipopolysaccharides are essential for the survival of bacteria, their release due to antimicrobial attacks lead to bacterial death (Powers & Hancock 2003).

In a study performed by Zhang et al. (2017), the mechanisms of antimicrobial activity of AMP derived from human milk were revealed through DNA binding, and scanning and transmission electron microscopy experiments. The researchers demonstrated that *S. aureus* and *Y. enterocolitica* cells treated with AMP exhibited substantial disruption in the bacterial cytoplasmic membrane and the cell wall of *Y. enterocolitica* was destroyed. Due to the bacterial anionic character of the cytoplasmic membrane and cationic of AMP, it kills bacteria by attracting anionic bacterial surfaces and separating polar and hydrophobic residues, facilitating its interaction with the peptide and its placement within the bacterial cytoplasmic membrane (Wang et al., 2014). Accordingly, it can be said that the antimicrobial activity of AMP is caused by cytoplasmic membrane disruption.

Similarly, in a study investigating the effect of β-casein obtained from human milk on *E. coli, S. aureus*, and *Y. enterocolitica*, wrinkled surfaces, bubble-like structures, destroyed membranes, and infiltrated cytoplasm were detected compared to normal morphology (Fu et al., 2017). However, β-casein has also been reported to have some other intracellular targets for killing bacteria, such as induction of autolytic enzymes or inhibition of proteins required for the bacterial life cycle.

The action mechanism is similar in whey-derived peptides. It is assumed that amphiphilic peptides disrupt the cytoplasmic membrane of microorganisms. The antimicrobial mechanisms of whey proteins are unclear. However, these products are thought to increase cell permeability and disrupt cell metabolism. The low ATP content in the presence of whey proteins support this concept. ATP affects the protein synthesis mechanism of the cells, resulting in lower bioluminescence emission. Therefore, peptides inhibit the growth and metabolic performance of pathogens (Pihlanto-Leppälä et al., 1999).

22.3 Dairy-derived organic acids

Organic acids are vital compounds produced as intermediate and end products of glucose catabolism, fat hydrolysis, and growth of bacteria during cheese manufacturing (Izco et al., 2002). Lactic, acetic, butyric, and propionic acid are the primary dairy organic acids and mainly formed during the ripening of many kinds of cheese. The amount of organic acid is essential for the nutritive value of the dairy product. At the same time, organic acids are also important for keeping bacterial activity under control, as they are important secondary carbon sources for bacteria (Akalin et al., 2002).

Murtaza et al. (2017) studied the effect of probiotic bacteria during cheese ripening and determined the effect of high ripening temperature on the concentration of organic acids (lactic, citric, acetic, and butyric acids). As a result, it was reported that a significant increase was observed in the concentrations of all organic acids during ripening in relation to the high temperature. This increase was more in samples containing probiotic bacteria such *as Lb. acidophilus, Bf. bifidum*, or *Bf. longum*.

22.3.1 Antimicrobial effect of organic acids

It is difficult to report a standard practice for studying the antimicrobial activity of organic acids. Besides, it is known that acids create an antimicrobial effect because of the low pH value of the product. Additionally, to compare the activity of different organic acids is difficult due to the impact of the physico-chemical structure, microbial species, and growth conditions (Cherrington et al., 1991). The main inhibitory mechanism of organic acids observed was a result of a pH-lowering effect.

Some researchers investigated the antimicrobial properties of different acids on pathogenic bacteria. Tejero-Sariñena et al. (2012) showed that production of organic acids especially lactic and acetic acid inhibit the activity of pathogens (*Sal. typhimurium, E. coli, Ec. faecalis, S. aureus* and *Clostridium* (*Cl.*) *difficile*). Similarly, antimicrobial properties of acetic acid, citric acid and lactic acid against four *Shigella* (*Sh.*) species such as *Sh. sonnei, Sh. flexneri, Sh. boydii* and *Sh. dysenteriae* were examined (In et al., 2013). In this study, it is stated that lactic acid inhibited the growth of all *Shigella* species. However, citric acid weakly inhibited the *Sh. flexneri* growth, while it strongly inhibited the growth of *Sh. dysenteriae*. Acetic acid exhibited the weakest antimicrobial activity among the tested organic acids. Hsiao and Siebert (1999) showed that while lactobacilli were much more resistant to acetic, benzoic, butyric, and lactic acids than other tested organisms (*B. cereus* (ATCC11778), *B. subtilis* (ATCC6633), *E. coli* (ATCC25922), *Lb. plantarum, Lb. fermentum* (ATCC14931), and *Alicyclobacillus*), *E. coli* was determined as the most resistant to citric, malic and tartaric acids. In another study, it was determined that in the neutral pH region (pH 6.5), caproic and caprylic acid had an antimicrobial effect against *E. coli* (Skřivanová & Marounek 2007). At lower pH, the effect of caproic acid remained similar, but caprylic acid further reduced the concentration of viable cells. The antibacterial activity of capric acid was low at pH 6.5 but increased at pH 5.3. Medium-chain fatty acids had a higher antimicrobial effect than other organic acids examined (C6-C14 fatty acids, oleic, citric, lactic, and fumaric acid). As a result, researchers indicated that the antimicrobial activity of fatty acids toward the C6 strain was pH-dependent.

22.4 Conclusion

Antimicrobial substances derived from milk are the most concentrated group of bioactive peptides. Many AMPs can be obtained from different raw materials with different methods. These peptides can be classified as coming from casein and whey. However, in this study, it was classified AMP was obtained enzymatically and microbiologically according to the production method. Since the main effect of AMP is to keep the activity of harmful microorganisms under control, it provides a protective effect against pathogenic bacteria, thereby an impact on health. As a result, it is effective on many infections as well as on oral and dental health. While the mechanism of action of AMPs is based on the damage of the cytoplasmic membrane, the effects of organic acids depend on the increase in acidity.

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Chapter 23

Bacteriocins and antimicrobial peptides as an alternative to antibiotics

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Abbreviations

AA	amino acids
AMPs	antimicrobial peptides
AMR	antimicrobial resistance
ARGs	antibiotic resistance genes
BLIS	bacteriocins like inhibitory substances
CDC	Centers for Disease Control and Prevention
ESBL	extended-spectrum beta-lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species
FMT	fecal microbial transplantation
GDP	gross domestic product
GRAS	generally regarded as safe
HGT	horizontal gene transfer
LAB	lactic acid bacteria
LPS	lipopolysaccharides
MBL	metallo-beta-lactamase
MDR	multidrug resistant
MDRE	multidrug-resistant enterococci
MRSA	Methicillin methicillin-resistant -*Staphylococcus aureus*
OECD	Organization for Economic Cooperation and Development
PG	phosphatidylglycerol
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization

23.1 Introduction

The discovery of antibiotics has been one of the greatest therapeutic milestones to cure infectious diseases of bacterial origin. An accidental discovery of penicillin and its subsequent introduction into the global market was the beginning of the so-called "golden era of antibiotics," which boosted the discovery of other antibiotics between 1940–62 (Luqman et al., 2020). However, shortly thereafter, the emergence of bacterial resistance towards developed antibiotics has substantially threatened the efficacy of antibiotic therapy. The enduring process of bacterial resistance towards last-resort antibiotics has continued to date with the emergence of colistin-resistant strains (Sherry & Howden, 2018). Of note, the alarming rise in the drug resistance pattern among pathogenic strains and their subsequent dissemination into the environment is an important public health threat of international concern. Unraveling the rationale behind such

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inevitable emergence of superbugs has highlighted the following underlying reasons: (1) indiscriminate or injudicious (over or sublethal dosage) use of antibiotics in the various fields of agriculture, veterinary, and human medicine; (2) lack of antibiotic stewardship; and (3) lack of discovery of new antimicrobials (Founou et al., 2016). Indeed, these factors have triggered microbial adaptation mechanisms towards different antimicrobials under selective pressure. The mechanisms such as the efflux pump, modification in the drug target site, decreased uptake of antibiotics, biofilm formation, and enzyme-mediated drug inactivation contributes to intrinsic resistance (Fig. 23.1). On the other hand, resistance is acquired only when susceptible microorganism imbibes antibiotic resistance genetic material from other organisms via horizontal gene transfer (HGT) routes viz. transduction, conjugation and, transformation (Sultan et al., 2018). However, conjugation is the major means of HGT, wherein the antibiotic resistance genes (ARGs), located on plasmids, display greater potentiality of lateral transfer across the genus and species (Bennett, 2008). On the other side, flanking of ARGs between the transposons or insertion (IS) elements may binate the genome reorganization process that changes the resistance patterns. Integron-mediated drug resistance is of paramount concern, since they carry multiple resistance genes and, thus, the overuse of less crucial antimicrobials may result in the selection of more critically important antimicrobials (Babakhani & Oloomi, 2018).

Recently, the global annual death due to antimicrobial resistance (AMR) was accounted 700,000 and it has been reported that the death toll would be expected to hike up to 10 million by 2050, which might lead to a decrease in the gross domestic product (GDP) by 2.5% (Ghosh et al., 2019). This death toll underscores that further use of antibiotics may annoy the AMR crisis. This necessitates the unraveling of novel antimicrobials that act through multifarious actions. To encourage research and development, the World Health Organization (WHO) had provided a *Global Priority Pathogens List* (PPL); a catalog of 12 multidrug-resistant pathogens against which alternatives to antimicrobials are highly warranted on a priority basis (Asokan & Vanitha, 2018). The experts have classified the pathogens into three priority tiers based on the species and type of resistance: critical, high, and medium. Microbes under the first priority (critical) are *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant Enterobacteriaceae, carbapenem-resistant, 3rd generation cephalosporin-resistant. Whereas, the microbes such as *Enterococcus faecium*, vancomycin-resistant *Staphylococcus aureus*, methicillin-resistant, *vancomycin*-intermediate and resistant *Helicobacter pylori*, clarithromycin-resistant *Campylobacter*, fluoroquinolone-resistant *Salmonella* spp., fluoroquinolone-resistant *Neisseria gonorrhoeae*, 3rd generation cephalosporin-resistant, and fluoroquinolone-resistant are a priority 2 (high). Likewise, *Streptococcus pneumoniae*, penicillin-nonsusceptible *Haemophilus influenzae*, ampicillin-resistant *Shigella* spp., and fluoroquinolone-resistant have been grouped under priority 3 (medium). Similarly, the Centers for Disease Control and Prevention (CDC) has classified a plethora of microbes as posing urgent, serious, and concerning threats, based on their substantial clinical and financial burden observed in the United States health care system (Fig. 23.2). Therefore the development of novel antimicrobials against multidrug- and extensively drug-resistant Gram-negative bacteria is a major goal of modern researchers. On the other hand, the WHO panel has urged for new antibiotics in the form of oral formulations for community diseases with a high morbidity burden, such as drug-resistant *N. gonorrhoeae*, *Salmonella typhi*, and ESBL-producing Enterobacteriaceae. It has been reviewed that several studies by hospitals and the Infectious Diseases Society of America have nominated nosocomial infections causing few pathogens (Gram-positive and Gram-negative) as ESKAPE pathogens, namely, *Enterococcus faecium*,

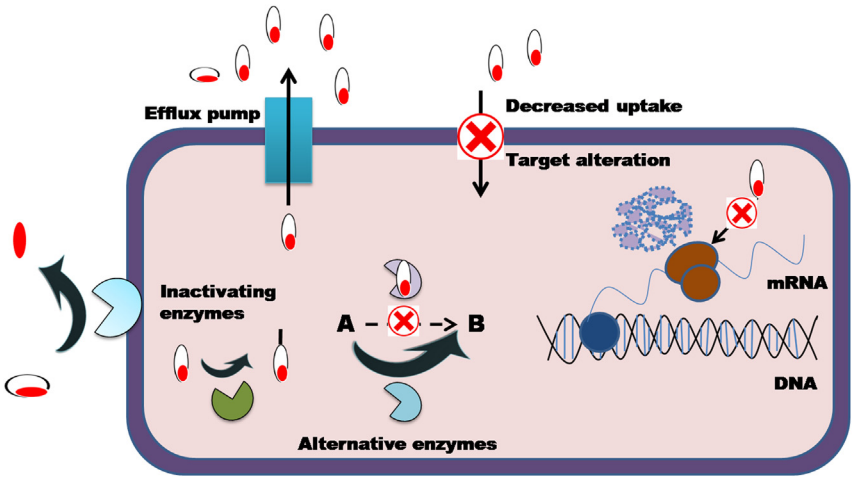

FIGURE 23.1 Diagrammatic representation of bacterial intrinsic resistance toward different antibiotics.

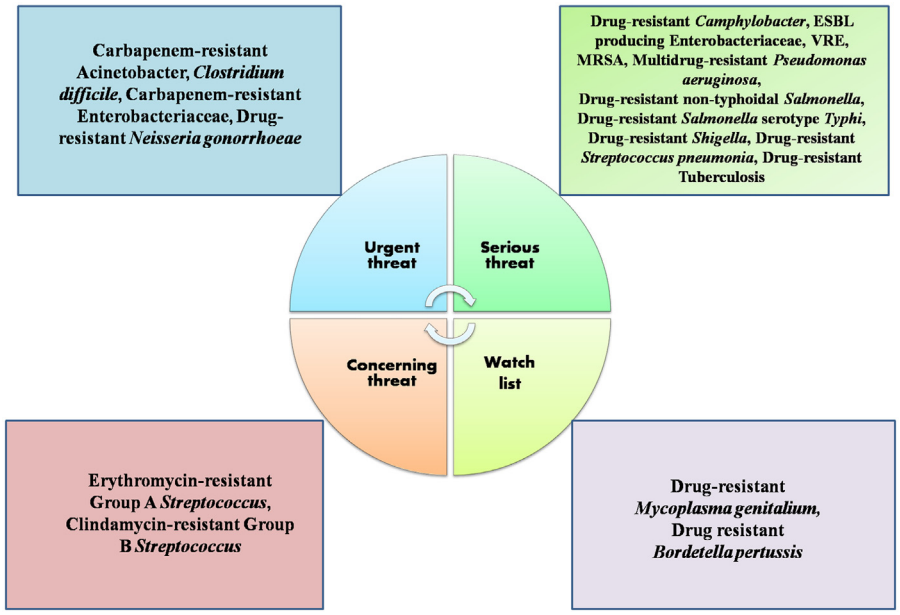

FIGURE 23.2 Classification of multidrug-resistant clinical pathogens according to Centers for Disease Control and Prevention (CDC).

S. aureus, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species (Songhita et al., 2020). Henceforth, multifaceted, comprehensive, integrated measures with interdisciplinary research efforts adhering to the one health approach are imperative to manage this contemporary pressing crisis.

23.2 Alternatives to antibiotics

As more and more antibiotics render as inefficient, interest is now gradually shifting to search for alternatives to conventional antibiotics, which shows less propensity to develop cross-resistance. In this regard, probiotics, prebiotics, postbiotics, antibodies, bacteriocins, fecal microbial transplantation (FMT), bacteriophages, endolysins, and antimicrobial peptides (AMPs) have been seen as alternatives to antibiotics (Ghosh et al., 2019). However, a few of their limitations have hampered the full potential of on-field applications. For example, probiotics or FMT are largely confined to decolonize AMR superbugs in the human gut. However, the mode of action of FMT or probiotics is largely general and fails to decolonize the targeted group of bacteria in the gut unless genetically modified (Koo et al., 2012). Moreover, the intervention of live probiotics or FMT increases the risk of lateral flow of ARGs from the donor (superbugs) to recipient (probiotics or FMT) cells. Similarly, the success of bacteriophage therapy has been hampered by its unclear regulatory requirements, narrow-spectrum activity, huge costs involved in clinical trials. Moreover, the safety of phage therapy is a debatable issue as they reviewed to show cytotoxicity and immunogenicity due to the presence of endotoxin and pyrogenic substances. More importantly, obtaining intellectual property rights is a difficult task for therapeutic phages for a variety of reasons, with varying patenting procedures in the United States and the EU (Ghosh et al., 2019; Henein, 2013). The phages that undergo only the lytic cycle are more desirable than the lysogenic cycle, since the lysogenic cycle of phages may act as a vector to transfer ARGs from one host to another (Mahony et al., 2011). Antibody therapy is another alternative to tackle resistant infections (Ghosh et al., 2019). Nevertheless, their high cost of production and poor shelf life have lowered their onsite application. Hence, the present chapter focuses on the potential and prospect of bacteriocins and AMP as alternatives to conventional antibiotics.

23.3 Bacteriocins

Every living organism produces proteinaceous antimicrobial substances for their survival. For example, eukaryotes produce AMP like defensins and cathelicidins which serve to fight against invading pathogens as the first line of defense. Similarly, prokaryotes also produce two types of AMPs to survive in the highly competitive polymicrobial environment: (1) those that are ribosomally synthesized (also known as bacteriocins) displaying a quite narrow range of antimicrobial activity, and (2) nonribosomally synthesized AMPs (exhibiting a wider range of activity towards bacteria or fungi), which are not encoded by structural genes (e.g., e-poly-L-lysine, lipopeptides, and glycopeptides) (Chikindas et al.,

2018). It is worth mentioning that the "true" bacteriocins, such as colicins and colicin-like bacteriocins that are so-called bacteriocin-like inhibitory substances (BLIS), differ from each other in several aspects such as structure, stability, mode of actions, etc. Hence, bacteriocins, in general, may be defined as ribosomally produced multifunctional substances of proteinaceous nature with pronounced antimicrobial activity at certain concentrations. Bacteriocins possess a net positive charge and amphipathic nature; however, there is even anionic bacteriocin (subtilosin A) reported in the literature (Noll et al., 2011; Thennarasu et al., 2005). Therefore stating bacteriocins as net positive charge (cationic) peptides might result in a contradictory statement. Bacteriocins are produced by a variety of both Gram-positive and Gram-negative bacteria in their vicinity for self-preservation and competitive exclusion of pathogens. Interestingly, a few members of the archaeal domain have also been reported to produce bacteriocin or proteinaceous antimicrobials such as archaeocins, halocins, sulfolobicins, etc. (O'Connor & Shand, 2002; Pašić et al., 2008). Although bacteriocins are the strenuous protagonist among the various microbial-derived antimicrobial compounds, they are effective only on the bacterial species that are closely related phylogenetically to host bacteria. The microbial genome mapping has displayed the location of bacteriocin synthesis genes either on chromosomal DNA or plasmids or transposons to encode structural proteins including immunity proteins to prevent self-toxicity (Flórez & Mayo, 2018; Mokoena, 2017; Perin et al., 2016). The bacteriocins of lactic acid bacteria (LAB) are gaining significant interest among food and pharmaceutical industries for several reasons: (1) "Generally Regarded as Safe (GRAS)" status accorded by the United States Food and Drug Administration; (2) safe or nontoxic to eukaryotic cells; (3) active against several foodborne spoilage and pathogenic bacteria; (4) does not interfere with the sensory quality of foods; (5) degraded by gastric proteases of the mammalian digestive system; (6) the ribosomal origin of bacteriocins has facilitated to manipulate the associated structural genes to obtain variants with more beneficial properties; and (7) specific pathogenic bacterial community can be focused, due to the narrow spectrum of certain bacteriocins, with no effect on commensal gut microbiota (Mills et al., 2011; Shin et al., 2016). Amid the diverse bacteriocins, nisin is the class II food preservative that has received regulatory approval by the European Union (E-number: E-234 as a food additive) and the US Food and Drug Administration (Malvido et al., 2019). However, studying bacteriocins as biotherapeutics to fight against MDR pathogens remains one of the hot areas of research since the development of alternatives to conventional antibiotics is indeed in a real emergency. To date, several studies have been conducted to evaluate the antagonistic actions of bacteriocins against MDR pathogens. Results suggesting the superior antimicrobial, antiadhesion and antibiofilm actions as presented in Table 23.1. Besides targeting membrane pore formation, the bacteriocins, such as thiopeptides and bottromycin, have been reported to affect the cellular translation process. Whereas, Microcin B17 (MccB17), MccJ25, and MccC7-C51 have been reported to alter the metabolism/normal functioning of DNA, RNA, and proteins (Ghosh et al., 2019).

23.4 Classification and mode of actions of bacteriocins

Bacteriocins are categorized based on the host organism, mode of action, molecular mass, physicochemical properties, and amino acid composition. Although bacteriocins are produced by both Gram-positive and Gram-negative bacteria, the majority of the reported bacteriocins of food applications belong to Gram-positive, and particularly LAB. According to recent literature, the bacteriocins produced by Gram-negative bacteria are classified into four types (Fig. 23.3) (Simons et al., 2020) and those produced by Gram-positive bacteria are grouped into four types as discussed next.

23.4.1 Class I bacteriocins

These are posttranslationally modified (cyclization of specific amino acid residues) lantibiotic peptides of 19−50 amino acids with molecular weight less than 5 kDa. These usually contain nonstandard or unusual amino acids, such as lanthionine, β-methyllanthionine, dehydrobutyrine, dehydroalanine, and labyrinthine-forming multiple ring structures that provide structural stability towards heat, pH, and proteolysis. Class I is further divided into class Ia (these are positively charged elongated lantibiotics such as nisin A and Z, epidermin, gallidermin, etc., and are usually associated with bacterial membrane pore formation by interacting with lipid II molecule or peptidoglycan transporter bactoprenol, and thus inhibiting cell wall synthesis as shown in Fig. 23.4.) (Islam et al., 2012), class Ib (these are negatively charged globular and inflexible labyrinthopeptins such as lacticin 481, cytolysin, salivaricin, etc.), and class Ic (sanctibiotics). Class 1b inhibits the target bacteria by interfering with the action of specific essential enzymes. On the other hand, lantibiotics can use lipid II as a tool to initiate the process of membrane insertion and pore formation (Kumariya et al., 2019).

TABLE 23.1 Antagonistic activities of bacteriocins against multidrug-resistant pathogens.

Source of bacteriocin	Targeted MDR pathogen	Mode of action	References
Lactocin XN8-A (*Lactobacillus coryniformis* XN8)	*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213	Antimicrobial activity (growth inhibition) by inducing membrane permeability and pore formation at MIC 6.85 mg/mL. XN8 showed cell cycle arrest at both G1 and G2/M phase	Yi et al. (2016)
Plantaricin GZ1−27	Methicillin-resistant *S. aureus* (MRSA)	Antibiofilm and antimicrobial activity at MIC 32 μg/mL. However, treatment of GZ1−27 at sub-MIC concentration showed expression of proteins responsible for biological process (biofilm formation, DNA replication and repair, and heat-shock) and metabolic pathways (purine metabolism, amino acid metabolism, and biosynthesis of secondary metabolites)	Du et al. (2020)
Enterococcus faecalis 28 and 93 (Enterocins DD28 and DD93) derived class IIb bacteriocins	MRSA	Antimicrobial and antibiofilm activity	Al Atya et al. (2016a)
Nisin and enterocin DD14	Colistin-resistant *E. coli*	Antimicrobial and antibiofilm activity	Al Atya et al. (2016b)
Nisin like bacteriocin-GAM217	Extended-spectrum β-lactamase (ESBL) and metallo-beta-lactamase (MBL) producing clinical strains of *E. coli*, *Klebsiella pneumonia, Proteus mirabilis*	Antimicrobial, antibiofilm, and antiadhesion ability	Sharma et al. (2020)
Bacteriocin Bac-SMO1 from *Bacillus subtilis* SMO1	MRSA, *Acinetobacter baumannii*, ESBL *E. coli*, *Pseudomonas aeruginosa*	Antibacterial activity	Mickymaray et al. (2018)
Lactobacillus crustorum MN047 derived bacteriocin BMP32	*E. coli* and *S. aureus*, *Cronobacter sakazakii*, *Salmonella*	Antibacterial activity	Qiao et al. (2020)
Lactobacillus pentosus derived pentocin JL-1	MRSA and *E. coli*	Antimicrobial activity	Jiang et al. (2017)
Bacteriocin (BAC-IB17) derived from *B. subtilis* KIBGE-IB17	MRSA	Antibacterial activity at the killing concentration 80 A/mL	Ansari et al. (2018)
E. faecalis EF478	Multidrug-resistant enterococci (MDRE) and vancomycin-resistant enterococci (VRE)	Antibacterial activity	Phumisantiphong et al. (2017)
Enterocin P	Vancomycin-resistant enterococci (VRE), and carbapenem-resistant *P. aeruginosa*	Antibacterial activity	Tanhaeian et al. (2019)
Durancin 61A derived from *Enterococcus durans* 61A	*Clostridium difficile*, vancomycin-resistant *Enterococcus faecium*, and methicillin-resistant *S. aureus*	Antimicrobial activity	Hanchi et al. (2017)
Bacteriocin MN047A isolated from *Lactobacillus* crustorum MN047	Drug-resistant MRSA and *E. coli*	Inhibiting the growth at G_1 and G_2/M phases	Yi et al. (2016)
Garvicin KS derived from *L. garvieae*	Drug-resistant *E. coli*, *P. aeruginosa*, and *Acinetobacter* strains	Antibacterial activity	Chi and Holo (2018)

(*Continued*)

TABLE 23.1 (Continued)

Source of bacteriocin	Targeted MDR pathogen	Mode of action	References
Pasteuricin derived from *Staphylococcus pasteuri* RSP-1	MRSA	Antibacterial activity	Hong et al. (2018)
Lactobacillus casei derived LiN333 bacteriocin	Drug-resistant *Salmonella typhimurium* and *S. aureus*	Antibacterial activity	Ullah et al. (2017)
Bacteriocin of *P. aeruginosa*	MRSA	Antibacterial activity	Arumugam et al. (2019)
Bacteriocin-like inhibitory substance (BLIS)	Vancomycin-resistant *Enterococcus* (VRE) strains	Antimicrobial activity	Shokri et al. (2014)
BLIS	Drug-resistant *Helicobacter pylori*	Antimicrobial activity	Boyanova et al. (2017)

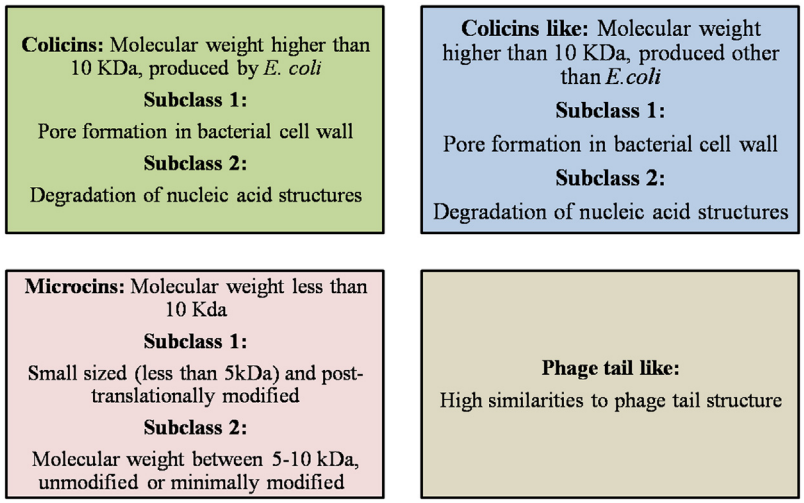

FIGURE 23.3 Classfication of bacteriocins derived from Gram negative bacteria.

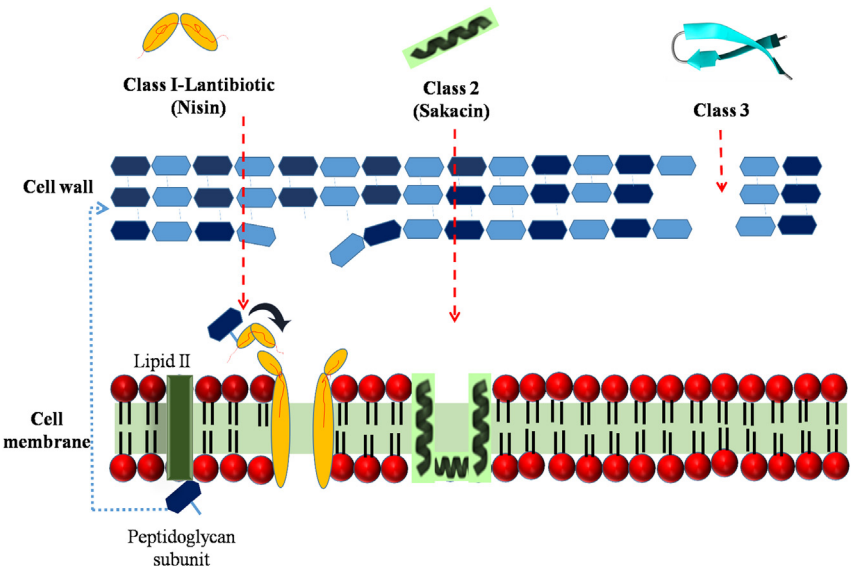

FIGURE 23.4 Diagrammatic representation of mechanistic action of different bacteriocins on bacterial cell wall and cell membrane.

23.4.2 Class II bacteriocins

These are small and nonlantibiotic containing peptides with (<10 kDa) with limited modified, membrane-active, and temperature- and pH-resistant polypeptides. The posttranslational modification is limited to disulfide bond formation in a few bacteriocins such as pediocin PA1 and pediocin AcH. The pediocin-like peptides cause destabilization and permeabilization of the bacterial membranes and, thus, induce membrane pore formation. The mature bacteriocins of this class have been reported to assume amphiphilic helices with wide-ranging hydrophobicity, and moderate (100°C) to high (121°C) heat stability (Rodali et al., 2013). These are further subclassified into four groups. The members of subclass IIa exhibit linear structure with disulfide linkages. Since these exhibit antilisterial activities, they are also termed as antilisterial bacteriocins (e.g., leucocin A, acidcin A, pediocin PA1) (Kumariya et al., 2019). Class IIa and a few class IId bacteriocins (lactococcin A and lactococcin B; linear, non-pediocin-like) target the mannose PTS system as a receptor (Cotter, 2014). Sequence alignment of class IIa bacteriocins showed the highly conserved hydrophilic and charged N-terminal end exhibiting the consensus sequence of YGNGV(X)C(X)4C(X)V(X)4A (X denotes any amino acid) with a variable hydrophobic and/or amphiphilic C-terminal part with pI values ranging from 8 to 10 (Rodali et al., 2013). Class IIb bacteriocins (lactacin F, lactococcin M, lactococcin G, lacotcoccin Q, plantaricin NC8) are two peptide bacteriocins (alpha/beta) that are paramount for demonstrating antagonistic activity. Class IIc bacteriocins are the leaderless bacteriocins, wherein the leader peptides contain one or two cysteine residues (cystebiotics and thiobiotics) which are cleaved upon modification to form peptides without an N-terminal leader peptide (lactococcin B). Class IIc bacteriocins are referred to as circular bacteriocins since N- and C-terminal ends are sealed to form a circular backbone that aids to withstand wide-ranging temperature and pH. The subclass IIc bacteriocins include lactoccin A, divergicin A, acidocin B, garvicin ML, etc. Maltose ABC transporter is the target for their activity by triggering the release of intracellular cellular constituents via the permease component of the transporter, or the maltose transporter may simply act as a docking molecule (Cotter, 2014). Class IId bacteriocins are unmodified nonpediocin-like linear bacteriocins and these usually includes the bacteriocins of Class II that are not included in the above classification (Kumariya et al., 2019). Overall, bacteriocins allocated in class II have amphiphilic helical structures that allow them to insert into the bacterial membrane and cause depolarization. On the other hand, Zn-dependent metallopeptidase was found to be a bacterial target which is responsible for sensitivity towards the class IId bacteriocin LsbB (Cotter, 2014).

23.4.3 Class III bacteriocins

These are the large heat-labile lytic or nonlytic proteins (>30 kDa) exhibiting an antibacterial knack corroborating the enzymatic activity of endopeptidase that disintegrates the cell wall architecture. Examples for class III bacteriocins include helveticin J, helveticin V-1829, lysostaphin, lactacin A and B, and acidophilus A (Rodali et al., 2013). Lysostaphin is a staphylococcal bacteriocin (Bastos et al., 2010) and colicin and microcins are by the Gram-negative species such as *E. coli* (Destoumieux-Garzón et al., 2003). Similarly, pyocin and salmocins are produced by *P. aeruginosa* and *Salmonella* species, respectively (Schneider et al., 2018). Lysostaphin catalyzes the hydrolysis of the peptidoglycan layer of Gram-positive bacteria and, thus, facilitating bacterial lysis. Whereas, colicins and microcins have demonstrated multifaceted inhibitory actions such as membrane disintegration, pore formation, inhibition of peptidoglycan synthesis, degradation of peptidoglycan layer, inhibition of DNA gyrase, inhibition of RNA polymerase, proton channel inhibition, and inhibition of proteosynthesis (Bosák et al., 2020).

23.4.4 Class IV bacteriocins

These bacteriocins are complex proteins displaying both essential lipid or carbohydrate moieties in their structure. However, these protein-macromolecule complexes are often referred to as bacteriolysins (hydrolytic polypeptides), and thus result in only three categories of bacteriocins. Nevertheless, the reclassification of bacteriocin is indeed an enduring process since R&D is a continuous process and data accumulates continuously regarding their intricacy and diversity. The examples for class IV bacteriocins include plantaricin S or leuconocin S. These disrupt bacterial cell membrane integrity which leads to loss of cell viability. However, the presence of both proteins and carbohydrate moieties makes the members of this class sensitive to several enzymes (i.e., glycolytic or lipolytic enzymes) (Rodali et al., 2013).

23.5 Antimicrobial peptides

AMPs are the amino acid sequences that are naturally produced by the multicellular organisms which are commonly called "host defense peptides" in eukaryotes (Nicolas, 2009; Prabhu et al., 2013). AMPs contribute to innate/nonspecific immunity, which confers resistance against infectious diseases without prior exposure to foreign pathogens (Chen et al., 2018). These peptides constitute a sequence of 5−40 amino acids with a molecular weight of less than 10 kDa (Palermo & Kuroda, 2010). These peptides function as natural antibiotics to curb the proliferation of invaded pathogens. Besides the antimicrobial property of AMPs, a variety of other functions such as skin regeneration, antineoplastic effects, antitumor, and wound-healing properties have been reviewed in the literature (Kosikowska & Lesner, 2016). AMPs exhibit broad-spectrum activity depending upon application such as antibacterial, antifungal, antiviral, antiplasmodial, antiprotistal, insecticidal, and immunomodulation activities. The major advantage of these peptides is that bacteria do not develop cross-resistance since the membrane disintegration is energetically unfavorable (Ghosh et al., 2019). Although AMPs are reported to exhibit a net positive charge (cationic nature), anionic AMPs have also been frequently reported in the literature (Harris et al., 2009). Since AMPs are amphiphilic, their cationic domain involves electrostatic interactions with the negatively charged bacterial cell surface, whilst the hydrophobic moiety interacts with the lipids of the bacterial membrane. This interaction perhaps disintegrates the bacterial cell membrane, finally leading to cell death (Palermo & Kuroda, 2010). It is worth mentioning here that due to the zwitterionic nature of mammalian cells, they fail to interact with the positively charged AMPs, and thus deemed them selectively toxic only to bacteria (Ghosh et al., 2019). Moreover, their natural antimicrobial properties, selectivity, speed of action, and a low propensity for the development of bacterial resistance make them ideal candidates for therapeutics. AMPs universally display similar fundamental properties such as amphipathicity and hydrophobicity (Kumar et al., 2018). Besides membrane pore formation, these peptides have been reported to affect various intracellular activities such as inhibition of DNA and protein synthesis, altering enzymatic activities, and abrogate cell wall synthesis and septum formation (Brogden, 2005). By contrast, the nonlytic and nonmembrane permeabilizing peptides have drawn substantial attention as promising antibiotics for therapeutic applications and disease control. The examples for nonlytic and nonmembrane permeabilizing peptides include polyphemusin of the horseshoe crab, pleurocidin of winter flounder, and tritrpticin of mammalian bone marrow (Nagarajan et al., 2018). According to the recent literature (Songhita et al., 2020), classical features of an excellent/ideal antimicrobial peptide are listed below:

- The size of the peptide should be 6−59 amino acids with anionic nature. The peptide sequence should contain basic amino acids like arginine/lysine, hydrophobic residues like alanine, leucine, and phenylalanine. The ratio of hydrophobic to a charged concentration should be 1:1 or 2:1.
- The preferable configuration should be α-helical. Some of the AMPs are found in the form of two antiparallel β-sheets (γ-core motif).
- Sufficiently hydrophobic to partition through the cell membrane.
- The peptide should be soluble in water and biological fluids for easy and quick transportation and accessibility to the target microbes (low hydrophobicity is required).
- The hydrophobic region of the peptide should be freely accessible to interact with the lipid bilayer to disturb and delocalize the membrane structure (high hydrophobicity is required).

Besides eukaryotic AMPs, peptide antibiotics derived from the bacterial source also show similar broad-spectrum activity. For example, polymyxin B (a lipopeptide obtained from *Bacillus polymyxa*), colistin (polymyxin E, also from *B. polymyxa*), gramicidin (a linear polypeptide derived from *Bacillus brevis*), daptomycin (a cyclic anionic lipopeptide)—and are being used in the clinics (Brandenburg et al., 2012; Kwa et al., 2007; Trimble et al., 2016). Based on the secondary structures of peptides, Boman's classification categorizes AMPs into grouped into four classes viz. α, β, $\alpha\beta$, and non-$\alpha\beta$. Likewise, the eukaryotic AMPs are classified into cationic (e.g., defensins, cathelicidins, cecropins, thionins) and anionic peptides (Maximin, dermcidin) (Nagarajan et al., 2018).

23.6 General classification of antimicrobial peptides

23.6.1 Type 1 (alpha-helical peptides)

The membrane-binding domain of alpha-helical peptides is composed of a linear arrangement of amino acids with repeated units (every three to four residues across the structure) of polar and apolar amino acids. The occurrence of polar amino acids on one side and the hydrophobic amino acids on the other side of the helix facilitate the appropriate

interaction of such peptides with the cell membrane. In fact, these are the most abundant cytolytic AMPs with an amphipathic and alpha-helical motif that aid in successful interaction with the cellular membrane (Huang et al., 2010; Wiradharma et al., 2011). It is important to note that hydrophobicity and helicity are two paramount factors that drive the potentiality and biological activities of these peptides (Zelezetsky & Tossi, 2006). D-amino acid substitution method to form D- and L-diastereomeric peptides is a well-known reported method to alter the hydrophobicity and helicity of peptides to binate the properties, such as peptide specificity, stability, better antimicrobial activity, and lower cytotoxicity against healthy or noncancerous cells (Nagarajan et al., 2018). The examples for these peptides include Cap11, Cecropin B, Melittin, Magainins, Cecropin P1, and Cap18. Cathelicidins are the mammalian cationic AMPs belonging to this class (Ebbensgaard et al., 2015). Cathelicidins contain the N-terminal cathelin domain and C-terminal cationic antimicrobial domain that becomes active after cleavage. Besides antimicrobial activities, it has been reported that cathelicidin can bind to endotoxin and neutralize its effects on the host. LL-37 and FALL-39 are the human cathelicidins and peptide antibiotics, which show a broad-spectrum inhibitory action against numerous bacterial, fungal, and viral pathogens (Parisien et al., 2008).

23.6.2 Type 2 (beta-sheet peptides)

These are the cysteine-rich cyclic peptides with 16−18 amino acids having a β-strands secondary structure that is widely present among the animal kingdom. These peptides are structurally stabilized by intramolecular cysteine disulfide bridges. Examples of these peptides include alpha and beta-defensins, tachyplesins, protegrins, bactenecin, and dodecapeptides (Miyasaki & Lehrer, 1998). These show inhibitory actions by disrupting the cell membrane via pore formation. Moreover, the antiviral properties against both enveloped and nonenveloped viruses via targeting the viral envelopes, glycoproteins, and capsids in addition to inhibition of viral fusion and postentry neutralization have been documented. Moreover, the alteration of host cell surface receptors by effective peptide binding and disruption of intracellular signaling by defensins can also inhibit viral replication (Wilson et al., 2013).

23.6.3 Type 3 (peptides with repeated units of few amino acids)

Only a few selected peptides belong to this class and are distinct from other classes due to the less abundance of typical secondary structure. The uniqueness of peptides in this class relies on their overrepresentation or unusual representation of few amino acids such as proline, tryptophan, histidine, and arginine (Nagarajan et al., 2018). Penaeidins is one such peptide exhibiting multiple proline residues in their N-terminal end and six cysteine residues at the C-terminal end (Gueguen et al., 2006). Similarly, Mytimacin-AF is a novel type-3 cationic peptide from a land-living mollusk *A. fulica*, which is composed of a total of 80 amino acid residues including ten cysteine residues (Jian et al., 2013). Histatins is another AMP of this class comprising a group of a small histidine-rich, cationic multifunctional peptide found in the human saliva, with less than 5 kDa molecular weight. Histatins represents typically a high content of histidine, which accounts for about 25% of all amino acids (Parisien et al., 2008).

23.6.4 Type 4 (looped peptides with single bond)

The typical peptides belonging to this class are known by their looped structure due to the existence of a single bond (disulfide or amide or isopeptide bond) or circling of the peptide chain (Rahnamaeian, 2011). Structurally, peptides of this class vary from the Type II peptides by the presence of only a single disulfide bond with antiparallel β-sheet orientation. Bactenecin and thanatin (21- amino acid residue antimicrobial peptide derived from *Podisus maculiventris* displaying a C-terminal disulfide loop) are examples of looped peptides. The small size, easy to synthesize, and proteolytically stable nature of these peptides indeed enabled them to consider as potential candidates to combat emerging infectious diseases (Nagarajan et al., 2018).

23.7 Mechanistic action of antimicrobial peptides

Although the exact mode of inhibitory actions of AMPs is yet to be unraveled; studies hitherto have explained that the antagonistic action of AMPs depends on their structural properties. Therefore their sequence, size, cationic nature, hydrophobicity, and amphipathicity propel their ability to crosstalk with target cells (Giangaspero et al., 2001). More clearly, it is the structural architecture of bacterial membrane i.e., negatively charged phospholipid head in the lipid bilayer makes bacteria vulnerable to AMPs (Verardi et al., 2011). On the other hand, eukaryotic membranes bear

predominantly neutral phospholipids (zwitterionic phospholipid), which as a result, are less susceptible to disruptive action of cationic peptides (Ghosh et al., 2019). To date, numerous models have been put forth to describe the cell membrane disruption modes by AMPs. Amid the several mechanisms, Shai-Matsuzaki-Huang (SMH) model (peptides adopt a three-dimensional structure upon the interaction with the bacterial membrane and fold into amphiphilic molecules, where the positively charged sides directly interact with the anionic lipid headgroups of the bacterial membrane), Barrel-stave model (the hydrophobic domain orient towards the membrane lipid portion, and the hydrophilic region forms the inside portion of the pore; example: Alamethicin and gramicidin S), Carpet model (peptides attach negatively charged cell membrane electrostatically in such a way that peptides are spread all over; example: Cecropin), Toroidal-pore or wormhole (the peptide insertion causes bending of the lipidic portions in such a way which gives a structure of pore; example: Magainin 2), and aggregate or detergent model (formation of combined peptide- membrane lipid-water transmembrane channel at the cell membrane leads to the formation channel for leakage of cytoplasmic content; e.g., Polyphemusin and indolicidin) have been reported to be predominant ones (Bobone et al., 2012; Hale & Hancock, 2007) as illustrated in Fig. 23.5.

The general steps involved in AMPs-mediated cell membrane disruption include attraction, attachment, and peptide insertion. In the initial phase of attraction, the electrostatic bonding facilitates the interaction between charged peptides and negatively charged bacterial surface (Hädicke & Blume, 2017; Henderson et al., 2019). Here, the peptide must penetrate deep into the polysaccharide bacterial surface and must interact with the charged cellular components such as the lipopolysaccharide, especially among Gram-negative bacteria or teichoic and lipoteichoic acid from Gram-positive bacteria. It was reported that the α-helical structures of AMPs are more effective since their attachment to the bacterial membrane can be accomplished at even low peptide/lipid ratios (Sato & Feix, 2006). Later, any of the aforementioned models may be implemented to disruption of the cell membrane integrity (Table 23.2). Despite pore formation, AMPs have also shown their intracellular killing activity by modulating few biochemical and cellular processes such as; (1) the upregulation of autolysins e.g., N-acetylmuramoyl-L-alanine which acts as an autolysin activator, (2) modulators of DNA replication like Buforin II (an antimicrobial peptide of 21 amino acid sequence), (3) halt in DNA, RNA, and protein synthesis by pleurocidin (antimicrobial peptide found in the mucus secreted by the skin of the winter flounder), dermaseptin (polycationic peptide of 20−30 amino acid length, secreted by the skin of amphibians that play an important role in defense mechanisms against a broad spectrum of pathogens), HNP-1 (peptide produced by human neutrophil possessing both broad antimicrobial proteins found in saliva), drosocin (AMP 19 amino acid chain derived from fruit fly *Drosophila melanogaster*), apidaecin (proline-rich AMP, which was isolated from the hemolymph of honeybees), indolicidin (bactericidal and permeabilizes the bacterial membranes) (Hale & Hancock, 2007; Songhita et al., 2020).

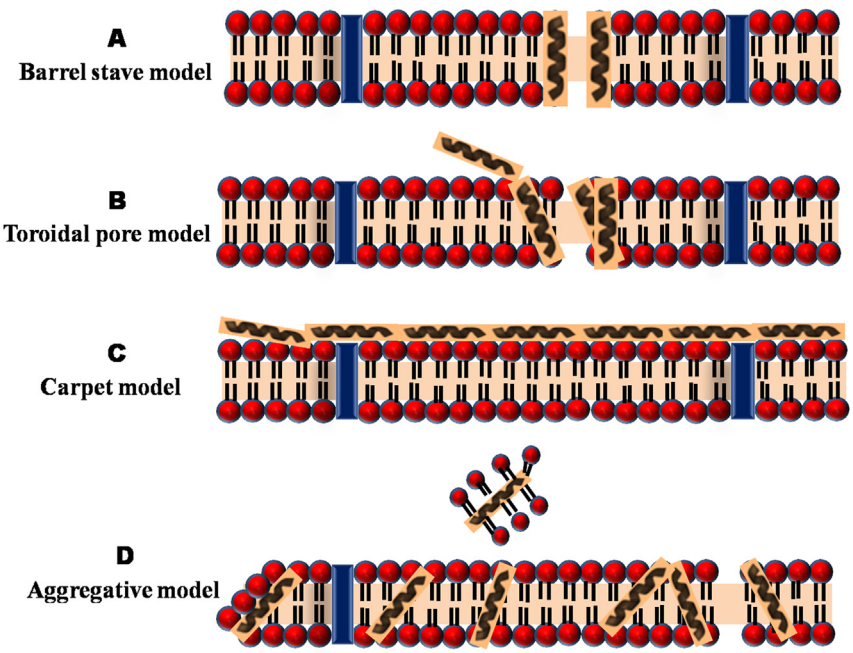

FIGURE 23.5 Different models illustrating the mode of action of antimicrobial peptides on bacterial cell membrane.

TABLE 23.2 Antagonistic actions of antimicrobial peptides against multidrug-resistant pathogens.

Type of antimicrobial peptide	Target pathogen	Mode of action	References
Synthesized antimicrobial peptide (FAKKFAFKKFAKKFAKFAFAF)	Drug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*	Antimicrobial activity by killing the growth or proliferation	Schwab et al. (1996)
Surfactin	Methicillin-resistant *S. aureus* (MRSA)	Antimicrobial activity via disruption of the bacterial membrane	Chen et al. (2015)
Cathelicidin antimicrobial peptide LL-37	Carbapenem-resistant isolates of *P. aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*	Bactericidal action	Lin et al. (2015)
Arg and Trp containing cationic antimicrobial peptides (RRWVRRVRRWVRRVVRVVRRWV RR)	MRSA, *A. baumannii*, *K. pneumonia*, *P. aeruginosa*	Antimicrobial activity	Deslouches et al. (2013)
LL-37: human cathelicidin, CAMA: cecropin (1−7)-melittin A(2−9) amide, magainin II and nisin	MRSA; multidrug-resistant *P. aeruginosa*	Antimicrobial activity	Geitani et al. (2019)
Melittin-derived peptides (i.e., MDP1 and MDP2)	MRSA, *Escherichia coli*, and *P. aeruginosa*	Antimicrobial activity via pore formation as well as cell lysis (DNA leakage)	Akbari et al. (2018)
AMPs (antilipopolysaccharide factor from shrimp, epinecidin-8 from grouper, and pardaxin-6 from *Pardachirus marmoratus*)	Methicillin-resistant *S. aureus*	Antimicrobial activity by pore formation	Lin et al. (2013)
Short synthetic β-sheet folding peptides, IRIKIRIK (IK8L), IRIkIrIK (IK8-2D), and irikirik (IK8D)	Multidrug-resistant *P. aeruginosa*	Membrane-lytic antimicrobial mechanism	Zhong et al. (2017)
α-helical peptides (WKKWWKKWWKKW-NH2 and WKKWWKKWWK-NH2)	MRSA and *K. pneumoniae*	Permeabilization of bacterial membranes and Antibiofilm activities	Khara et al. (2017)
Tridecaptins are produced by *Bacillus* and *Paenibacillus* species	*A. baumannii* and *K. pneumoniae*	Antimicrobial activity	Ballantine et al. (2019)
α_{s1}-casein derived peptides viz. IKHQGLPQE, VLNENLLR, and SDIPNPIGSENSEK.	*Enterobacter sakazakii* ATCC 12868 and *E. coli* DPC5063	Antimicrobial activity	Hayes et al. (2006)
NuriPep 1653 (a novel 22 mer and nonmodified peptide) which is involved in nutrient reservoir activity in *Pisum sativum*	Pan drug-resistant *A. baumannii*	Antimicrobial activity	Mohan et al. (2019)

23.8 Food-derived antimicrobial peptides

Microbial fermentation of food or gastrointestinal enzyme-based protein digestion results in an array of peptides with biological activities. The peptides with 2−30 amino acids are baptized as bioactive peptides (Mohanty et al., 2016). However, several foods naturally contain antimicrobial factors that curb the growth of microbes such as milk, honey, etc. (Evison et al., 2016; van Hooijdonk et al., 2000). The peptides of <5 kDa size derived from soya fermentation showed antimicrobial activity against *Vibrio parahaemolyticus* (Cheng et al., 2017). Likewise, several milk-derived bioactive peptides have portrayed inhibitory action against Gram-positive and Gram-negative bacteria (Nielsen et al., 2017). Amongst the several bioactive peptides, AMPs are the amphipathic peptides having low molecular weight (<2 kDa) with 2−10 amino acids, bearing a net charge of +2 to +9 (Shivanna & Nataraj, 2020). Amino acids such as Pro, Arg, Val, Leu, Glu, Cys, Ala, and Lys are predominant in AMPs (Sah et al., 2018). Cell membrane disruption by electrostatic interaction, alteration in membrane permeability, and/or inhibition of protein, DNA, and RNA synthesis have been the probable mode of action of food-derived AMPs on target bacterial cells (Daliri et al., 2018). The

amphipathic nature of AMPs coupled with the cationic nature of peptides disrupts cell membrane integrity. The hydrophilic and cationic peptides interact with the negatively charged components of the cell membrane (LPS or LTA). By contrast, the hydrophobic domain of the peptides transacts with the lipid bilayer to alter its integrity (Mohanty et al., 2016). Several AMPs have been generated by enzymatic degradation of αS1-casein. These include f (1−23), f (10−14), f (1−7), f (6−14), f (15−22), and f (180−193) (Sah et al., 2018). On the other hand, Isracidin f (1−23), Casocidin-I f (150−188), Casocidin-I f (181−207), Casocidin-I f (175−207), Casocidin-I f (165−170), lactoferricin f (17−41), lactoferrampin f (265−284) Casocidin-I f (165−181), Kappacin f (106−169), and Kappacin f (42−49) have been other AMPs derived from bovine milk (Mohanty et al., 2016). Indeed, such peptides have been found successful to curb the growth and proliferation of both Gram-positive and Gram-negative antibiotic-resistant bacteria (Abdel-Hamid et al., 2020).

23.9 Synthetic designed peptides

Advent in molecular medicine has paved the way to generate molecules to mimic the AMPs. Moreover, the tunable nature of these synthetic peptides aid to tackle the hurdles of protease lability, poor in vivo activity, toxicity, and the high cost of manufacture of AMPs (Ghosh et al., 2019). Hence, to overcome such limitations posed by natural AMPs, several synthetic peptides have been designed and are currently under the different phases of preclinical and clinical trials (Table 23.3). It is important to design AMPs demonstrating superior scaffold designs with enhanced pharmacodynamic and pharmacokinetic properties such as better inhibitory actions with minimal mammalian toxicity. The major strategies in peptide synthetic science include improving the protease stability by altering the peptide backbone without changing the basal cationic and amphiphilic structures. It is because the cationic peptide (the net positive charges traverse between +2 to +9) has strongly correlated with its antimicrobial activity (Takahashi et al., 2017). Hence, incorporation of hydrophobic and cationic moieties into the synthetic polymers would certainly result in polymers with better antimicrobial activity. On the other hand, peptide length plays a crucial role in the safety or toxicity of the AMPs. It was previously reported that too long or high lipophilic peptides exhibit high toxicity against human RBCs. Henceforth, the peptides manifesting appropriate length (9−12 residues) and lipophobic/lipophilic balance might demonstrate higher biological activities (Takahashi et al., 2017). These are classified into three classes: polymeric mimics of AMPs (these mimic the amphiphilic alpha-helical arrangement of side chains as in the natural AMPs); peptidomimetic oligomers (beta-peptides, arylamide oligomers, phenylene ethynylene oligomers, oligoureas, peptoids, oligoacyllysines, alpha-AA peptides); and small molecules (these are the AMPs inserted with facial amphiphilicity into small molecules via hydrogen bonding) (Ghosh et al., 2019; Som et al., 2008). The cationic AMPs interact with the outermost glycocalyx and/or cell wall surface of bacteria and fungi that includes various anionic components such as murein, lipopolysaccharides (LPS), phosphatidylglycerol (PG), and cardiolipin (Som et al., 2008). The mode of action of these synthetic mimic AMPs is similar to that of naturally occurring peptides as previously explained with different models.

23.10 Safety aspects of bacteriocins and antimicrobial peptides

Since several antimicrobials have been commercially applied, it is paramount to evaluate the safety of bacteriocins in various experimental models before human applications. Additionally, the potential to develop bacterial counter-resistance towards bacteriocins on repeated administration is necessary to evaluate to avoid treatment failure in the near future. Fig. 23.6 addresses various steps involved in the identification, characterization, and safety evaluation of bacteriocins. The various safety tests include cytotoxicity against eukaryotic cells (LDH, neutral red, and MTT-assays in non-cancerous cell lines), gastrointestinal stability and absorption (stable to gastric pH and proteases), immunogenicity (should not be allergenic), and impact tight junction integrity (the integrity of epithelial tight junction should not be altered when assayed through in vitro HT-29 or HCT-8 or Caco-2 cell lines/in vivo models) (Samira et al., 2021) (Table 23.4). On the other hand, the impact of bacteriocins on the gut microbiome (modulation in the abundance of commensal microflora) has been studied and found that the overall beneficial gut microbial imprints remained largely unaffected upon the treatment with sakacin A (SakA), pediocin PA-1 (PedPA-1), enterocins P, Q and L50 (enterocins), plantaricins EF and JK (plantaricins) and garvicin ML (GarML). By contrast, potentially harmful bacteria were inhibited (e.g., *Staphylococcus* by enterocins, Enterococcaceae by GarML, and *Clostridium* by plantaricins) and the proportion of LAB was increased in the presence of SakA-, plantaricins-, and GarML-producing bacteria. Moreover, the treatment with GarML-producing bacteria co-occurred with decreased triglyceride levels in the host mice (Umu et al., 2016). Similarly, the intestinal wall disruption, erythrocytes and lymphocytes toxicity, free radical production, enzymopathic, and immunopathic tissue damage, and cytotoxicity in suitable models are some of the other parameters to be

TABLE 23.3 Status of antimicrobial peptide molecules.

Name	Description	Medical use	Stage of development	Company
CSA (cationic steroid antibiotics)	The CSA share structural features in common with the antimicrobial peptides (AMPs) that form part of the body's innate immune system	Antiinfective, including multidrug-resistant organisms such as *Pseudomonas*, MRSA, VRSA	Preclinical	Ceragenix
Plectasin	Fungal defensin	Anti-Gram-positive especially Pneumonococcal and Streptococcal infections	Phase 1	Novozymes, Denmark
Mersacidin	Bacteriocin like	Gram-positive and MRSA infections	Preclinical	NovactaBiosystems Ltd.
LL-37	Human (neutrophils and epithelial cells)	MRSA	Preclinical	Inimex Pharmaceuticals
IMX942	Synthetic cationic host defense peptide derived from IDR-1 and indolovidin	Nosocomial infections	Phase 2	Inimex
Novexatin (NP213)	A cyclic cationic peptide derived from NovoBiotics arginine peptide platform	Treatment of dermatophyte fungal infections	Phase 2B	Unknown
PMX-30063 (Brilacidin)	Defensin structural mimetic, nonpeptide, small molecule	*Staphylococcus aureus* infections	Phase 2	PolyMedix
Pexiganan acetate (MSI-78)	Synthetic cationic host defense peptide (22 mer), maganin derivative	Topical antibiotic	Phase 3	MacroChem
Mureparadin (P0L7080)	Synthetic by amino acid substitution of protegrinI	Treatment of nosocomial penumonia and ventilator-associated bacterial pneumonia	Phase 3	Polyphor Ltd.
Omiganan (MBI-226/ MX-226/ CLS001)	Synthetic 12 mer cationic peptide derived from indolicidin	Treatment of atopic dermatitis	Preclinical	Maruho Co., Ltd
Buforin II	Asian Toad (*Bufo gargarizans*) stomach	*S. aureus* infections	Preclinical	Unknown
Heliomocin variants (ETD151)	An antifungal 44 AA peptide	MDR bacterial infections	Preclinical	EntoMed SA

Source: Data obtained from DRAMP database. http://dramp.cpu-bioinfor.org/browse/ClinicalTrialsData.php.

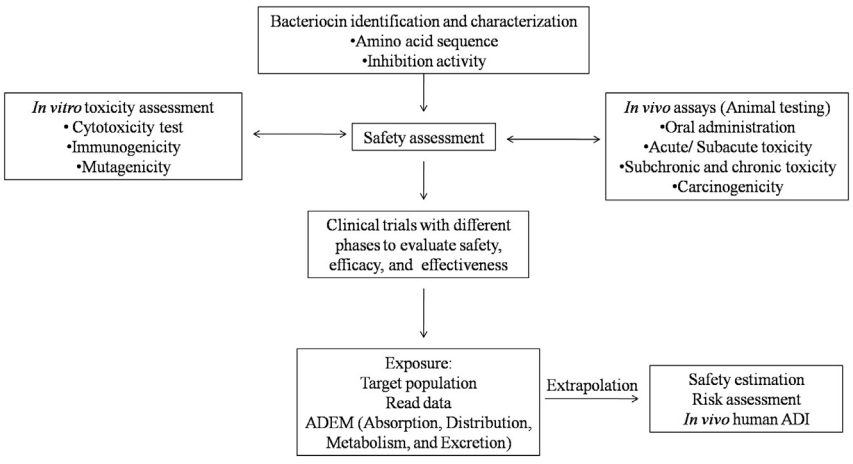

FIGURE 23.6 Road map for identification, characterization, and safety assessment of bacteriocins.

TABLE 23.4 Outcomes of in vitro cytotoxicity assessment of bacteriocins and antimicrobial peptides in different eukaryotic cell lines.

Bacteriocin or antimicrobial peptides	Cell line	Type of assay	Toxicity	References
Bacteriocin from *Bacillus subtilis* GAS101	Vero cell line	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay	No toxicity at 0.5 × MIC	Sharma et al. (2018)
Bacteriocin from *Lactobacillus sakei* GM3	HT-29 cells	MTT assay	No toxicity at 350 AU/mL concentration	Avaiyarasi et al. (2016)
Bacteriocin CV7	HeLa and HT-29 cell lines	MTT assay	Nontoxic at 100 AU/mL concentration	Perumal and Venkatesan (2017)
BacteriocinK2a2−3 derived from *Pediococcus acidilactici*	HeLa and HT-29 cell lines	MTT assay	Nontoxic at 800 AU/mL concentration	Villarante et al. (2011)
Bacteriocin from *Lactobacillus casei* TA0021	human lung fibroblast (MRC5) cell lines	MTT assay and hemolysis	Nontoxic at 0.25−2 μg/mL concentrations	Noroozi et al. (2019)
Bacteriocin AS-48	Human cell lines and in vivo mice and zebrafish embryos models	Hemolytic assay, zebrafish embryos acute toxicity assay, MTT assay on nontumor epithelial cells MCF10A and colonic fibroblast cells (CCD18Co)	Nontoxic up to 27 μM concentration (absence of lymphocyte proliferation in vivo after skin sensitization in mice, low hemolytic activity, and does not induce nitrite accumulation in nonstimulated RAW macrophages)	Cebrián et al. (2019)
Trypan blue exclusion assay	Nisin and pediocin	Nontoxic at 170 AU/mL	Vero cell line	Murinda et al. (2003)
Gallidermin, nisin A, natural magainin peptides, and melittin	HT-29 and Caco-2	MTT conversion assay, neutral red dye uptake assay	Gallidermin was the least cytotoxic AMP followed by nisin A, magainin I, magainin II and melittin at their IC50 concentration	Maher and McClean (2006)
Bombinin H4 and temporin A AMPs	NSCLC cell lines A549 and Calu-3 and normal epithelial cell line Beas-2B	CellTox green cytotoxicity assay and hemolytic activity	No toxicity at 1.5−25 μM concentrations	Swithenbank et al. (2020)
Cationic Antimicrobial Peptides (LL-37: human cathelicidin, CAMA: cecropin(1−7)-melittin A(2−9) amide, magainin II and nisin)	IB3−1 and A549 cell lines	LDH assay	Nontoxic at 64 μg/mL and 16 μg/mL concentrations	Geitani et al. (2019)
Pug-1, Pug-2, Pug-3, and Pug-4 synthetic nonlytic peptides	HaCaT cell line	MTT assay and Glucosyltransferase (Gtf) activity	Nontoxic at 25−200 μg/mL concentrations	Kokilakanit et al. (2020)
Plantaricin E and F derived from *Lactiplantibacillus plantarum*	Caco-2 cell line	Transwell permeability assay	PlnE and PlnF were required to prevent sustained cytokine-induced losses to Caco-2 cell para- and transcellular permeability	Heeney et al. (2019)

TABLE 23.5 Overview on toxicity assessment of bacteriocins and antimicrobial peptides in animal models.

Bacteriocins	Type of test	Concentration/dosage	Outcome	References
Enterocin AS-48, a circular bacteriocin produced by *Enterococcus* strains	Subchronic toxicity test in BALB/c mice	50, 100, and 200 mg/kg in the diet for 90 days	No abnormalities or clinical signs on body weights, food consumption, urinalysis, hematology, or blood biochemistry.	Baños et al. (2019)
Bacteriocin AS-48	Preclinical (acute toxicity) study in BALB/c mice	100 μg/mouse for 7 days	Absence of lymphocyte proliferation in vivo after skin sensitization in mice, and the lack of toxicity in a murine model. Moreover, no remarkable changes in serum biochemical measurements (% variation of uric acid, urea, creatine kinase-muscle/brain, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and bilirubin).	Cebrián et al. (2019)
Bacteriocin TSU4 from *Lactobacillus animalis* TSU4.	Immunogenicity, acute and subchronic toxicity	50, 100, and 200 mg/kg/body weight for acute test and 0.5 mg/kg/day dose of bacteriocin TSU4 for subchronic toxicity test	No mortality was observed during acute or subchronic toxicity tests. The LD_{50} value of bacteriocin TSU4 was found to be higher than 200 ± 0.45 mg/kg. No significant change in the serum biochemical markers, histopathological analysis and visual observation in spleen sizes was observed	Sahoo et al. (2017)
Antimicrobial peptide P34	Immunogenicity, acute, and subchronic toxicity in BALB/c mice	82.5, 165.0, 247.5, and 330.0 mg/kg body weight for 21 days. Whereas, 0.825 mg/kg/day of the peptide P34 for subchronic toxicity	No hypersensitivity reactions or a significant increase in antibody titer during the immunogenicity experiment or death of animals during the acute or subchronic toxicity tests. The LD_{50} was higher than 332.3 ± 0.76 mg/kg	de Almeida Vaucher et al. (2011)
Pediocin N6	Acute toxicity in White mice	5000 mg/kg to 20,000 mg /kg body weight of mice for 15 days	The LD50 value of Pediocin N6 was greater than 20,000 mg/kg	Marlida et al. (2016)

evaluated to unravel the safety status of AMPs (Khan et al., 2018). In the second phase, preclinical animal studies are conducted to evaluate the safety of test compounds before human clinical trials. Moreover, animal studies aid to establish the mode of action of test compounds and their appropriate dosage for further studies. Due to the absence of specific established guidelines for safety evaluation of bacteriocins or AMPs in animal models, the Organization for Economic Cooperation and Development (OECD) guidelines on safety aspects of chemicals in rodents are commonly employed (Samira et al., 2021). The outcomes of such trials upon administration of bacteriocins/AMPs have been compiled in Table 23.5. Accordingly, an acute toxicity test is carried out to evaluate the single-dose effect (50% lethal dose) of the test compound in a particular animal species for 14 days. Similarly, a subacute oral toxicity test is conducted for 28 days only upon gathering the tolerable dosage in acute toxicity testing. Subchronic toxicity is generally carried out for 90 days, wherein the information regarding health hazards occur upon subjection to a specific test compound after oral administration is gathered. By contrast, chronic oral toxicity is conducted to unravel the long-term effect (12 months) of a test substance in animals. In all the tests, the animals are examined for normal physiological functions, behavioral variations, alterations in biochemical parameters, and histopathological analysis is carried for the targeted tissues (Shivanna & Nataraj, 2020).

23.11 Conclusion

The global emergence of antibiotic-resistant infections associated with nosocomial and foodborne pathogens necessitates the development of novel antimicrobial agents. The developed antimicrobials should possess multifaceted action for their successful applications in therapeutics to effectively combat MDR superbugs. To date, numerous alternatives to conventional antibiotics have been developed to combat the threat posed by resistant bacterial infections. Amongst these, bacteriocins and AMPs have been found safe with a better therapeutic index and have shown the potential to curb the growth of resistant bugs. They exhibit multiple inhibitory actions such as membrane pore formation, antibiofilm activity, inhibiting cell wall biosynthesis, and affecting cellular metabolism. Moreover, the ribosomally synthesized AMPs or bacteriocins are amenable to the bioengineering strategies that can binate their activities against specific resistant bacterial strain. However, further studies are warranted to develop suitable delivery mechanisms, since the activity of bacteriocins may be affected by proteolytic digestion.

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Chapter 24

Nanobiotechnology in fermented dairy products

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24.1 Introduction

Since the initiation of civilization, milk has been used by humans for nutrition. The dairy product includes milk and foods made from milk. In many countries, half of the milk produced is utilized as pasteurized and skim milk. Milk products are commercially processed into more stable processed foods such as butter, dried milk, cheese, ice cream, and condensed milk. All through the world, cow milk is used as the primary type along with buffalo, goats, sheep, and reindeer. Generally, the technology used for cow milk is also applied to milk obtained from other animals. Fermentation is conducted to convert carbohydrates to alcohol or organic acids by bacteria or yeasts under anaerobic conditions. *Streptococcus* sp. and *Lactobacillus* sp. produce cultured milk by fermentation, in which lactose is converted to lactic acid. The shelf life of the milk product, taste, and digestibility are enhanced by fermentation. Common fermented dairy products available in the market are cultured buttermilk, yogurt, and sour cream. Other products like kefir, acidophilus milk, and milk products containing *Bifidobacteria* are rich in calcium and protein. So they provide numerous health benefits to the human diet, as well as maintain beneficial intestinal bacterial flora, and reduce lactose intolerance (Behera & Panda 2020). Dairy products, such as fermented whey beverages, fermented milk, and yogurt produced by traditional fermentation methods contain probiotic cultures. Nowadays consumers are looking for animal-based cholesterol-free fermented milk products because of lactose intolerance or high total cholesterol (Behera & Panda 2020; Sahu & Panda, 2018). Fermented products like cheese and butter have been used to preserve the nutrients of milk, to make it more transportable and digestible and readily available. This is due to the breakdown of lactose during fermentation. Since the 6th millennium BC, this type of milk processing has continued which has made significant progress early in the dairy farming industry (Salque et al., 2013). Due to the presence of micronutrients, proteins, and calcium, dairy products play an important role in the regulation of bone homeostasis (Rizzoli et al., 2014). Prebiotics like inulin, when added to yogurt, increases density whereas probiotics influence calcium absorption (Weaver et al., 2015). Kefir, a traditional probiotic fermented milk beverage is produced by kefir grains, composed of polysaccharides and proteins, and regarded as a nutritious and healthy dairy food (Joseph & Bachhawat 2014). However, by modern microbiological techniques, different fermented milk products are produced with higher nutritional value. These products characterize an essential component of functional foods. Several research projects are in progress to develop dairy products with the incorporation of probiotic organisms to add more value to the product (Parmjit, 2011). Nowadays, many dairy products like skimmed milk, paneer, full cream milk, toned milk, shrikhand, and many varieties of chocolates and sweets have been produced by fermentation. Consumers are attracted to fermented milk products due to new food processing techniques and scientific authentication of health benefits because of certain ingredients (Stanton et al., 2001). Bioghurt, yakult, actimel, etc. are examples of some cultured dairy foods which are already marketed as curative and dietetic products. In the area of food nanotechnology, research is in progress for food packaging and production of food supplements through applications of nanomaterials and their unique functions. Nanotechnology deals with matters that range from sizes of one-half the diameter of DNA to 1/20 the size of a red blood cell (Dingman, 2008). Most of the research based on nanomaterials are powders of nanoparticles. In the food and dairy industry, applications of nanotechnology include increasing the flavor of the food, encapsulation, delivery of substances to targeted sites, enhancement of shelf life, introducing antibacterial nanoparticles into food, improve food storage, sensing contamination, and brand protection (Neil and Scott, 2005) (Table 24.1). They also remove

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TABLE 24.1 Different nanoparticles and their uses in dairy and food industry.

Sl no	Types of nanoparticles	Name of nanoparticles	Function
1		Silver(Ag)	Antimicrobial food packaging
2		Zinc Oxide (ZnO)	Food packaging, UV absorbers
3	Inorganic	Iron Oxide (Fe_2O_3)	Food colorant, sources of bio available iron
4		Titanium dioxide (TiO_2)	Increases lightness and brightness
5		Silicon dioxide (SiO_2)	Anticaking agents to enhance flow properties i.e., sats, icing sugar
6		Lipid nanoparticles	Beverage emulsions
7	Organic	Protein nanoparticles	Delivering nutrients to infants
8		Carbohydrate (starch, cellulose)	Affect human health by altering bioavailability of encapsulated substances

chemicals and pathogens from food to enhance the nutritional value. Nano food packaging materials repair tears in packaging and provide a great barrier in packaging to increase food safety by releasing preservatives to prolong the life of the food. This also alerts consumers that food is spoiled. For the safety labeling of food products, nanobarcodes are also used. By encapsulation techniques, nanosupplements can be effectively delivered (Chellaram et al., 2014). The current chapter deals with the involvement of nanoparticles by nanotechnology in fermented dairy products to enhance the quality, taste, and storage period of different products.

24.2 Application of nano (bio)technology in dairy industry

Nanomaterials are manufactured and used on a small scale. Nanoparticles can be formed naturally, incidentally, or can be engineered. Crystal growth in the different chemical circumstances of the earth's crust produces natural inorganic nanomaterials; whereas, natural organic nanomaterial includes wax of a flower or leaf, natural colloids, horny materials (skin, hair, and horns), and even our bone matrix. Incidental nanoparticles are produced by engine exhausts from the vehicle, welding fumes, combustion processes from domestic cooking, among others. Engineered nanomaterials called fullerenes have been manufactured by humans (Portela et al., 2020); they are produced by burning gas, biomass, and candle (Barcelo & Farre 2012), which are then used in optoelectronics, having optical and electrical properties (Zeng et al., 2014). Recently, nanoparticles are being investigated for biomedical uses including drug delivery and tissue engineering (Kerativitayanan et al., 2015; Valenti et al., 2016). In healthcare, nanomaterials called nanozymes have enzymatic properties which are used (Wei & Wang 2013). They are a promising category of an artificial enzyme, which are used for biosensing, bioimaging, tumor diagnosis (Juzgado et al., 2017). Nanomaterials can exist in single or aggregated forms with tubular, spherical, and irregular shapes. Nanomaterials are of concern because at this scale unique magnetic, optical, and electrical properties emerge. Depending upon the size, morphology, physical, and chemical properties, nanomaterials can be classified; examples are metal nanoparticles, polymeric nanoparticles, lipid-based nanoparticles, etc. In the field of nanotechnology, nanomaterials display special physical-chemical features as normal chemicals (i.e., silver nano, fullerene, carbon nanotube, photocatalyst). Nanomaterials are used for storage life sensors, additives, package materials, clarification of products in the food and dairy industry (Fig. 24.1). Food nanotechnology is on the rise and opens up innovative opportunities for the food industry. Currently, the basic applications of nanotechnology focus on the development of food packaging. Nanotechnology plays a very important role in dairy processing due to food additives and food packaging. In the food industry, nanotechnology is in use to detect bacteria in packaging, production of flavor, and color. It also increases the barrier properties for food safety. This will help to design foods for diabetic people, which will be advantageous to maintain a balanced state of glucose release by eliminating the possibility of under- or overdosing. Specific nanoparticles are also used in the removal of pathogenic bacteria before they release toxins. The global rule for the regulation of nanotechnology in food is not well defined. Moreover, the existing rule appears inappropriate to nanotechnology specificity (Sekhon, 2010). Nanoparticles in the food help to develop the functional attributes of the food (Shekhon, 2010). Through nanotechnology, the crystalline structures in starch control the gelatinization and nutritional property of processed starch-based foods (Rudolph, 2004). Chinese nanotea is nothing but

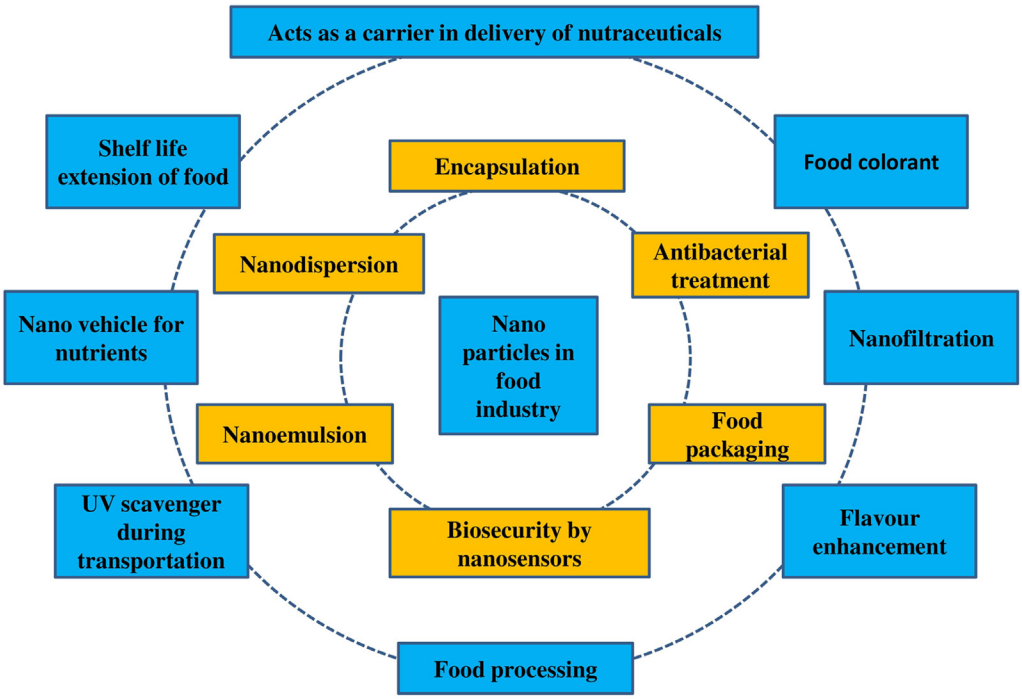

FIGURE 24.1 Different uses of nanoparticles in food industry.

nano-based mineral supplements that helps to improve selenium uptake (Yang & Koo 1997). Some of the nutraceuticals' built-in phytosterols, beta-carotenes, and lycopene are also known to avoid the build-up of cholesterol (Qureshi et al., 2012). In this chapter special emphasis is given to the involvement of nanotechnology in the improvement of dairy products. The dairy industries are in a technological revolution. To get the advantage and to continue leadership in the food and food processing industry, new cutting edge technology is required. Among all novel frontier technology, nanotechnology has earned great attention in the dairy industry. Many dairy products prepared with nanoengineered materials are being introduced into the market. Scientists are exploring the prospect of nanotechnology to encapsulate and deliver nutrients to targeted tissues for the enhancement of the flavor and other characteristics of foods. They are also motivated towards the introduction of antibacterial nanostructures into foods. In dairy industries, the applications include nanomaterial delivery systems such as nanodispersions and nanocapsules; in packaging, nanolaminates are used; in bins and bottles, nanocomposites are used; nanosensors are used for food safety. (Chen et al., 2006). So the potential benefits are not only in foods but also in food packaging and food processing, as well as food and nutrition science research. The engineering of nano foods needs basic food components like protein, fat, carbohydrates through various approaches (Morris & Parker, 2008). Nanotechnology would play a fundamental role in dairy processing in the near future. In 2003, the Dutch dairy company Campina launched Vifit Calcimel in the Netherlands which is calcium-enriched (Bijman, 2018). A cow's udder is the most interesting natural system for self-assembling nanostructures. It is an inspiring example of a biological nanodevice, where nature uses this approach of synthesis of proteins and fat in the aqueous phase. Later they develop building blocks for a large number of proteins. This further helps to form yogurt. Many aspects of nanoscience related to the dairy industry are discussed here.

24.3 Enhancement of the survival of novel microorganisms and nutraceuticals

Nanoparticles are very helpful in the delivery of drugs, vitamins, colorants, flavorings, and preservatives at a required concentration to preferred sites. These constituents are hardly ever utilized directly in their unadulterated form and often associated with some other forms for the delivery systems. Nanodispersion and nanoencapsulation are the best delivery systems as they include biopolymeric nanoparticles for nanoemulsion (Weiss et al., 2006). Several bioactive compounds such as vitamins, enzymes, pigments, flavors, essential fatty acids are present in dairy products. However, these bioactive compounds are susceptible to degradation due to heat, oxygen, light, and other stress conditions (Assadpour & Jafari 2019). In encapsulation at the scale of a nanometer or micrometer, one molecule is embedded into another

(Burgain et al., 2011). As several bioactive compounds are having less solubility, nanoencapsulation enhances the delivery (Bazana et al., 2019). During digestion, encapsulation helps to protect bioactive compounds and increase their uptake in the gastrointestinal tract (Zanetti et al., 2018), prevent the reaction with oxygen, light, etc., as they have the capabilities to deteriorate the food. The encapsulation techniques include extrusion method, spray drying, spray chilling, emulsion, coacervation, cocrystallization, among others. Different encapsulating agents such as polysaccharides (starches and maltodextrins), lipids (stearic acid and monoglycerides), and proteins (casein, milk serum) used for spray drying (Kavitake et al., 2018). Thangaraj and Seethalakshmi (2015) used an extrusion technique to encapsulate vitamin C in flavored milk. In this method, sodium alginate solution was prepared by adding L-ascorbic acid to the solution. Then the solution containing L-ascorbic acid was dropped into a calcium chloride solution to harden the vitamin-C microcapsules which were screened and washed with water. The mechanism of the emulsion technique is highly dependent on discontinuous and continuous phases. A little portion of the aqueous phase contains an emulsifier which is homogenized in a large portion of oil to outline a water-in-oil emulsion. The emulsion technique is a multipart process but produces smaller beads and is simple to scale up. Ascorbic acid which is found in milk in low content loses its major content during heat treatment of milk. To achieve the shortcomings, microencapsulation has been anticipated as a suitable approach (Lakkis, 2007) to make fortified milk products that are equipped with ascorbic acid and iron. Dairy products with encapsulated mineral salts were initiated to preserve different physicochemical and organoleptic properties of the product to develop novel purposeful foods. Microencapsulation of probiotic cultures in food is the best option to enhance the functionality of probiotics (Doraisamy et al., 2018). A study conducted by Dimitrellou et al. (2019) reveals that probiotic *Lactobacillus casei* ATCC 393 cells are encapsulated in Ca-alginate capsules showed high survival rates in fermented milk. Curcumin is a natural phenolic compound having anticarcinogenic, antioxidant, and anti-inflammatory properties. Due to the low solubility in water, the milk matrix which contains amphiphilic casein micelles provides a suitable medium for encapsulation of curcumin. Unsaturated-omega-3 fatty acids are important in the diet are prone to oxidation can degrade the nutritional quality of food, so the addition of encapsulated omega-3 fatty acids can trounce these troubles. Encapsulation of bioactive compounds in dairy products provides distinct advantages as it improves the stability of the compounds. Usually, food emulsion is produced by using food ingredients and techniques like homogenization (Weiss et al., 2006), similarly, nanoemulsion is produced by micro fluidizers or high-pressure valve homogenizers. By this, the ingredients can be incorporated at interfacial regions, droplets, or continuous phases (McClements, 2015). Nanoemulsion has distinctive rheological properties which considered them transparent and satisfying to touch due to its small droplet size (Sommerville et al., 2000). Multiple encapsulating capabilities from a single delivery structure can offer nanostructured emulsions due to complex properties. Nowadays, it is possible to build well-groomed delivery systems by manufacturing the nanostructured shell around the droplets (Qureshi et al., 2012). It can assist the use of less fat without finding the middle ground in creaminess for calorie conscience people. Such a thought is incorporated in the manufacture of ice-cream by Nestle and Unilever. In milk and milk-based products, a range of organoleptic characteristics like mouthfeel, savor, aroma, uniformity, and rheological distinctiveness is considered as the quality determinants. The attainment of preferred quality parameters in a product can be done by controlling the droplet size and distribution assisted by the emulsification procedure. The stabilizing capability of emulsion in milk is possible due to the natural emulsifying capacity of milk proteins. The advantage for nanoemulsions over traditional emulsions is growing day by day. Nowadays research is carried on meticulous applications and properties to improve the bioavailability of nutrients and enhance the physical stability of beverages and foods. Dairy-based products can be used for health and nutritional benefits. Milk protein (β-lactoglobulin) is denatured by various factors like heat, pressure, change in pH; then they are compiled and yogurt is formed. Nanotubes are developed by hydrolyzation of β-lactoglobulin and α-lactalbumin (Bugusu et al., 2009). Homogenization creates a favorable environment for the production of nano-engineered products. For example, the making of butter, creams, and yogurts by emulsification involves the production of gas bubbles or fat droplets in a liquescent medium. This requires an oil-water or air-water interface which determines its stability by its molecules. These structures, one molecule thick, represent two-dimensional nanostructures. The constancy of dairy froths and emulsions are controlled by two-dimensional (2D) nanostructure twisted at oil-water and air-water interfaces (Rudolph, 2004).

24.4 Flavor enhancements used as delivery systems for colors, flavors, preservatives, nutrients, and nutraceuticals

There is anxiety about the direct amalgamation of nanoparticles into foods used to generate flavors, preservatives, nutrients, and nutraceuticals. Organic (carbohydrate, lipid, protein) and inorganic (zinc oxide, iron oxide, silicon dioxide,

silver) molecules act as nanoparticles in foods. Nanoscale materials are present in casein micelles or certain cell organelles of plant or animal (Holt et al., 2003; Livney, 2010). Engineered nanoscale materials may be used as delivery systems for nutraceuticals, nutrients, flavors, and preservatives to modify the appearance and stability of foods. Nanoparticles used in foods are chiefly composed of silver, iron oxide, silicon dioxide, or zinc oxide (Pietroiusti et al., 2016). Silver (Ag) nanoparticles used in foods act as antimicrobial agents (Hajipour et al., 2012) whereas ZnO nanoparticles are utilized as antimicrobial agents (Sirelkhatim et al., 2015). Iron oxide (Fe_2O_3) as nanoparticles are applied as colorants in food or as a source of iron (Raspopov et al., 2011). TiO_2 particles are used in certain foods to enhance optical properties (Weir et al., 2012). Organic nanoparticles composed of lipids, proteins, or carbohydrates behave differently at different parts of the human digestive system. Generally, inorganic nanoparticles are more toxic than organic ones as they are not bio-persistent and are frequently completely consumed inside the human gastrointestinal tract.

24.5 Nanocarriers of nutraceuticals and therapeutic agents

The nano version of nutraceuticals are nanoceuticals, named by combining nutrition and pharmaceutical. The "nanoceuticals" are gaining a reputation in dairy and food supplements containing nanoparticles. Nanoceuticals are regarded as a mainstream product for the food and dairy market. Nanoceuticals are nutrients that have been manufactured by nanotechnology into nanoparticles. Dietary supplements are produced from nanoparticles by transforming fat-soluble nutrients into water-soluble ones, to make them more permeable through the cell membrane. In the nanotechnological blizzard, casein micelles, whey proteins, and fat globules play a very useful role in the dairy industry to create nanosized structures to achieve advantageous dairy products. For example, food proteins like α-lactoglobulin, can undergo denaturation and then reassemble to form larger structures, further gathered to form better gel networks to form yogurt. It acts as a probable new transporter for nutrients encapsulation (Bugusu et al., 2009). Casein micelles act as nanovehicles for the transfer of responsive nutraceuticals inside other food products (Semo et al., 2007). Nanocarriers have been developed to carry materials, which are unpredictable in the digestive tract after oral administration. For this, encapsulation is needed. Different bioactive components are entrapped in small capsules in nanoencapsulation, which allows them to be stable against nutritional losses during the process. Encapsulation of nutraceuticals increases their bioavailability, concentration, and food stability. Nanoparticle carriers are an efficient encapsulation method that protects them from environmental degradation agents. For example, casein micelle acts as a vehicle for nutraceuticals to deliver calcium phosphate and protein to the neonate due to its biological actions and good digestibility. For this, a classic nanosensor "electronic nose" is used for quality control of milk. Milk proteins are the most significant nanovehicles in food technology, as they help to carry hydrophobic nutraceutical substances to create emulsion-based carrier systems that are generated due to surface activity of caseins (Kimpel & Schmitt 2015). Recently, nanocarriers have been used extensively for the enhancement of long-term stability of different vitamins in fermented products as well as in the gut digestive system (Xia et al., 2014). Similarly, the nanocarriers have been used for the actual absorbance of vitamins from digestive systems as well (Khan et al., 2018).

24.6 Detection of adulteration and spoilage

Nanosensors and nanobiosensors take first place as an alternative to the classical methods used for ensuring the safety of foodstuffs. The nanoparticle-based nanosensors, electrochemical nanosensors, nano-tube based nanosensors, optical nanosensors, quantum dots, electronic nose, nanofibers, electronic tongue, and nanobarcode technology have significantly added to the food recognition practices in food systems with their sensing capabilities. Many applications like the detection of chemicals, biological contaminants, and adulterants are done by nanosensors to develop the quality and safety of food. Nanosensors also help in the food packing to protect foods alongwith improvement in thermal and mechanical properties. These sensors also monitor the freshness in raw and processed products, and the use of food additives like aroma and coloring agents. A dimethylsiloxane microfluidic immunosensor incorporated with a specific antibody, proven to be immobilized on an alumina nanoporous membrane, was developed for quick recognition of foodborne pathogens *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Similarly, FePt alloy nanoparticles can be used as a sensor for detecting vitamin C levels in food products (Moghimi et al., 2015). Aspartic acid-functionalized gadolinium oxide nanorods have been used for detecting vitamin D3 levels in food products (Chauhan et al., 2019). Ochratoxin-A (7-[L-β-phenylalanylcarbonyl]-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin, OTA) an abundant food-contaminating mycotoxin that is present in many food products. Nano-ZnO film has been deposited onto a glass plate along with other specific molecules that have been used to detect OTA present in the food materials (El-Ansary & Faddah 2010).

24.7 Food packaging

Currently, nanocomposite is used to reduce the adverse effects of artificial polymers for food packaging. Nanotechnology targets active packaging which can detect food adulteration and helps in shelf-life extension of food. This is involved in the improvement of smart packaging with a focus on indicators like humidity and freshness. Coatings by nanoparticles improve the barrier characteristics of packaging films. Improved packaging involves the integration of nanomaterials into polymers that improves packaging to protect the food from antimicrobial or ultraviolet scavengers. It includes smart packaging to identify biochemical changes in foods, track food safety, and prevent food adulteration. This technology also aims to widen bio-based packaging to decrease material waste and increase food quality. The dairy industry employs three ultimate nanosized structures (fat globules, casein micelles, whey proteins) to build all sorts of whipped cream, emulsions (butter), cheese, complex liquids (milk), and gel complexes (yogurt) (Aguilera & Stanley 1999). Nanofilters ensure the elimination of viruses and bacteria from milk to improve safety and shelf life. This is further enhanced by the use of silver-substituted zeolite products used in milk containers and paper food wraps. The most vital nanomaterials used in food packaging are zinc oxide (ZnO-NPs) coated silicate, montmorillonite (MMT), kaolinite, titanium dioxide (TiO_2NPs), and silver NPs (Ag-NPs). In these, nanomaterials coated films act as a barrier against O_2 and CO_2 and flavor compounds (Chaudhary et al., 2020).

24.8 Nanofilteration

Nanofiltration is a pressure-driven filtration process. Due to transmembrane pressure difference and porous type of membrane, both convective and diffusive fluxes happen during transport through the membrane. In dairy, new types of membranes like nano-sieves are applied. The pores of the sieves are in the range of nanometers. They can be utilized for cheese production by filtering the milk. Nanofiltration (NF) is a substitute for fractional demineralization which increases the nutritional importance of the product. NF membranes appropriate for the dairy industry have great permeability for salts like NaCl, KCl and little permeability for compounds like lactose, urea, and proteins. Nanofiltration is used to develop virus safety. Adding glycine may increase the artificial aggregation of viruses, which enhances the efficiency of virus removal (Buchacher & Curling 2018). Recently, it has been recommended that the involvement of small pore size (10−20 nm) nanofilters can remove prions (Buchacher & Curling 2018).

24.9 Safety and health implications

Since nanotechnology is a contemporary development, the health and safety aspects of publicities to nanomaterials and the heights of exposure are issues of ongoing research. Animal studies specify that carbon nanotubes cause pulmonary effects (Warheit et al., 2004). Engineered nanomaterials are used in cosmetics, foods, paints, fabrics, electronics, and fabrics. Nanotechnology deals with the food industry with many new methods for refining the shelf life, quality, healthiness of foods, and safety (Fig. 24.2). Lactoferrin, an iron-binding protein, present in milk, has the ability not only to slow down the growth of pathogenic bacteria, but also to separate bacteria from surfaces and prevent the reattachment of bacteria, so it helps in food safety. Nevertheless, there is an alarm from supervisory agencies, consumers, and food trade about the possible adversative effects (toxicity) related to the staging of nanotechnology in foods. Countless nanoparticles are not likely to have undesirable effects on human health; still, there are facts that some of them could have adverse properties, and for this upcoming studies are necessary. Food-grade nanoparticles occur as individual particles or may form groups that differ in size, appearance, and strength. As a result, to realize the destiny of ingested nanoparticles in the digestive tract and their possible toxicity, further research should be continued.

24.10 Regulatory

Under the sunshade of the Government of India's Nano Mission, nanomaterials-related safety has been an increasing alarm among the research and the regulatory agencies in India. The Nano Mission has decided to create a regulatory agenda to address the issues of the Environment, Health, and Safety (EH&S) impact and the probable risk arising from nanomaterials. the Centre for Knowledge Management of Nanoscience and Technology (CKMNT) has taken up new ideas to prepare guidelines for scientists in research and development laboratories to execute best practices for the safe management of nanomaterials at their workplaces. Looking ahead at the potential technical risks related to nanomaterials, there is only limited information available about the impact of nanomaterials on ecosystems. There should be guidelines to the researchers on the safe handling of nanomaterials for the safety of people as well as the ecosystem. In

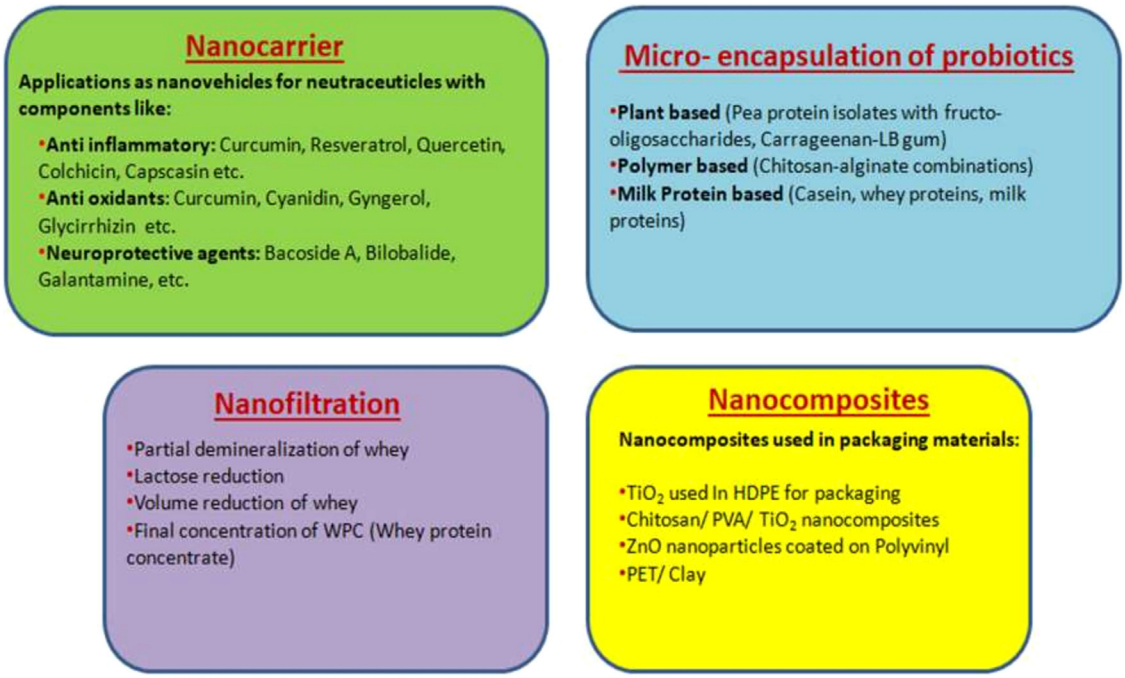

FIGURE 24.2 Major applications of nanotechnology in dairy industries.

the United States, there is no centralized legislation specific to nanomaterials. Nanomaterials are used in pesticides, food, chemicals, cosmetics, and drugs. In the European Union, nanomaterials are undergone by the same regulatory framework that ensures the safe use of all chemicals. This means that the hazardous properties of nanoparticles have to be assessed for their safe use. There are also specific requirements for nanomaterials in sector-specific legislation such as in the food industry.

24.11 Future direction of nanotechnology in fermented dairy foods

Few questions such as, How do the properties of nanomaterials alter when introduced into various types of food products and in food packaging? What happens when nanomaterials enter into the human gut? What is mandatory for evaluating and balancing the latent benefits and risks of introducing nanosized particles into foods and then into the human body? These types of questions need to be answered. Solving these problems requires coordinated, interdisciplinary efforts among engineers, chemists, and microbiologists working in the food sector, and others related to food products. Current food submissions of nanotechnology, security aspects of nanomaterials, routes of nanoparticles inflowing the body, prevailing policy of nanotechnology in several countries, and a documentation structure of nanoproducts have been previously described (Nicholas & Dervan 2007; Sozer & Kokini 2009). New characteristics of nanomaterials offer several opportunities for the dairy industry. Nanofibrils produced from milk fat globules were known to enhance viscosity efficiently, hence, they may have potential as a new thickener for food applications. Various functional nanostructures like nanoliposomes, nanoemulsions, nanoparticles, and nanofibers may be used as building blocks for creating new functionalities into foods and cause a great revolution in the dairy industry.

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Chapter 25

Application of nanomaterials in the dairy industry

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25.1 Introduction

Nanotechnology is one of the most rapidly progressing technologies in the world and has the greatest potential of becoming a boon to mankind. This technology has been an instrumental in various sparking progress across a multitude of industries, in order to improve our daily lives. These industrial sectors include automobile, pharmaceuticals, agriculture, water purification, energy, catalysis, food, etc. Therefore, nanotechnology is one of the most promising technologies to achieve the goal of reducing risk factors and improving the quality of life. It is mainly used in dairy food industries to improve product quality, shelf life and packaging (Cushen et al., 2012).

Food nanotechnology has contributed substantially to prevent the downfall of food and human sicknesses. Dairy products play a vital role in providing essential nutrients to the human body, particularly to the older age groups. Dairy products comprise 42.9% of the market share, with most s taken up by milk, cheese and yogurt (Kwak et al., 2014). Milk contains 0.9% minerals along with elements like sodium, calcium, phosphate, potassium, magnesium, chloride and sulfate (Gordon, 2014). Despite having benefits of several vitamins and minerals, there has been continuous efforts to improve its nutrient availability.

However, the rising concern among health-conscious consumers regarding the quality and safety of dairy products has increased exponentially (Santillán-Urquiza et al., 2017b). Consequently, the size of the materials is reduced to increase the surface area to volume ratio and eventually, elevate their efficiency (Lok et al., 2007). Nanomaterials are materials that fall under the nanoscale of 1−100 nm and exhibit characteristic features that are not offered by materials in their original form. These materials are exploited to enhance the quality, antimicrobial properties, delivery of bioactive compounds, nutritional value, packaging, shelf life, and safety of dairy products (Nile et al., 2020). The nanomaterials used in improving these characteristics are metal nanoparticles, nanorods, nanotubes, nanoemulsions, liposomes, quantum dots, nanosensors, nanocapsules, nanolaminates, nanocomposites, and so on. Different types of nanomaterials are responsible for dealing with different aspects of dairy products (Chen et al., 2006). Over the years, microbial contamination has been a major cause of the spoilage of dairy products. Metals such as silver, zinc, titanium, and copper were used to inhibit microbial growth, but were not as effective as they should be. Therefore, the emergence of metal nanoparticles brought the microbial contamination under control. This is because of the extraordinary antimicrobial properties and large surface area of metal nanoparticles, which prevent significant populations of microbes from expanding (Gong et al., 2007). Furthermore, metal nanoparticles, nanorods and nanotubes are also used to preserve the quality of dairy products.

Liposomes are nano-sized, single or multi-layered molecules that are made up of phospholipids and are used extensively in dairy sectors as a delivery agent (Lasch et al., 2003). Additionally, liposomes and micro- or nanocapsules are structures that encase bioactive compounds such as vitamins, minerals, carotenoids or poly-unsaturated fatty acids and deliver them to the target site while dairy processing (Chen et al., 2013). These molecules also provide protection against digestive enzymes or harsh environments that tend to dissolve the bioactive compound, as well as controlled release of the encapsulated compounds (Mozafari et al., 2008). Casein micelles are naturally occurring single-layered

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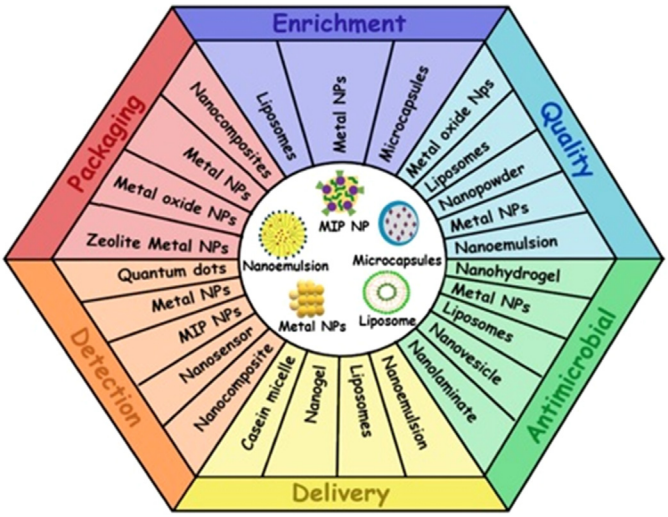

FIGURE 25.1 Graphical representation of various nanoparticles used in different sectors of dairy industry.

liposomes made up of proteins that transport calcium phosphate (Bhat et al., 2016). Similar to liposomes, nanoemulsions are another form of delivery agent that are manufactured using two immiscible liquids (emulsion) and are used for delivery of active ingredients (Panghal et al., 2019).

Nanolaminate is another type of nanomaterial, also known as laminate film, that is embodied with two or more nano-dimensioned layers that are principally bonded by chemical forces (Afroz et al., 2012). These nanomaterials are incorporated with flavonoids, antimicrobial agents, enzymes and coloring agents to improve the taste and shelf life of dairy products. They are also regarded as edible nano-coatings, as they do not cause any harm to our health and are far more advanced than standard technologies that are currently in use (Weiss et al., 2006). Furthermore, the most vital step in dairy processing is removal of harmful substances that are responsible for the early spoilage of dairy products. Nanosensors are used to detect harmful microorganisms, toxins, temperature, and pH. It is worthy in noting that, these sensors can be combined with other nanomaterials to enhance their performances (Bouwmeester et al., 2009).

Therefore, this chapter comprehensively focuses on the involvement of nanotechnology in dairy sectors that provides a potential aid to the novel approaches brought up to improve certain aspects of dairy products with characteristic properties. Furthermore, the current chapter discusses the sensory and physicochemical properties, and antimicrobial analysis of dairy products using nanomaterials to enrich quality control, antimicrobial activity, safety, detection, delivery of bioactive compounds, and packaging of dairy products (Fig. 25.1).

25.2 Application of nanomaterials in dairy industries

25.2.1 Nanomaterials used to increase the nutritional value

Nanomaterials have been exclusively used in dairy sectors to increase the nutritional value of dairy products. Metal nanoparticles alter the properties of dairy products while, in some cases, liposomes or microcapsules are used to deliver nutrients causing a rise in the nutritional value.

25.2.1.1 Iron, calcium, and zinc nanoparticles

Yogurt is a highly consumed dairy product yet, ironically, it has less amounts of iron, calcium, and zinc content; hence, an experiment was performed to fortify yogurt using metal nanoparticles (NPs). A study was conducted to differentiate between micro and nanoparticles, where all three metals were synthesized by the coprecipitation method and coated with inulin. Characterization using X-ray diffraction XRD (high crystallinity of Zn, the crystal size of Ca-80 nm, 47.1 and 47.7 nm of Fe), FTIR (presence of inulin), and transmission electron microscopy (TEM) (50−80 nm) were performed, followed by in vitro digestion (showing high solubility of NPs). After preparing the samples and keeping them under observation for 28 days, Santillán-Urquiza et al., observed no major changes in pH and acidity at the beginning, yet in the course of 28 days, pH gradually decreased (4.65−4.30) and acidity increased (0.86−0.90 g/100 mL). The obtained results showed that, syneresis was high in microparticles (Ca_{30M}-46.52, Zn_{50M}-51.18, and Fe_{50M}-55.39), proving stability of NPs. Furthermore, rheological properties revealed that in the presence of Zn and Ca NPs, consistency of

yogurt was higher, but Fe NPs did not show any major difference which proved that Ca_{30M} and Zn_{50M} NPs were more suitable in all aspects of fortification (Santillán-Urquiza et al., 2017a).

25.2.1.2 Nano-liposomes

Over the years, nano-liposomes have been useful in the enrichment of dairy products and the following experiment investigated the utilization of nano-liposomes for nano-encapsulation of fish oil for the fortification of yogurt. Nano-liposomes of size 300−500 nm (DLS) were manufactured by modified-homogenization methods. Subsequently, samples were prepared by adding 15 mL nano-liposomes to 100 g yogurt and were kept under observation for 21 days (7−14−21) at 4°C. Upon obtaining the results, Ghorbanzade, et al., reported an encapsulation efficiency of 92.22 ± 0.19% and liposome stability of 70%. Under physicochemical properties, the pH levels, syneresis, and peroxide values experienced decrement during the storage period and on the other hand, an increment in acidity was observed, due to the production of lactic acid that gradually decreased with time. There were no substantial changes reported in the sensory properties (taste, color, and texture) of nano-encapsulated fish oil. Furthermore, the DHA and EPA quantities in yogurt containing nano-encapsulated fish oil (57% and 12%) were more than yogurt containing nonencapsulated fish oil (27% and 6%). Therefore, this report also proves that involvement of nanotechnology in dairy products has positive impacts (Ghorbanzade et al., 2017).

Furthermore, enzymes are added to dairy products, especially cheese, to fasten their rate of ripening or maturation. Law and King used multilamellar vesicle (MLV) liposomes (produced by rotatory evaporation method) with size distribution from 0.25 to 5.0 μm and mean diameter of 1.68 μm (Fig. 25.2) to encapsulate proteinase, i.e., neutrase 1.5 S, thereby preventing the breakdown of substrates or casein molecules in cheddar cheese. Therefore, neutrase encapsulated liposomes were added to the cheese by two methods: (1) adding nonencapsulated Neutrase to cheese, and (2) adding liposome encapsulated neutrase to cheese. It was observed that the liposomes encapsulated was 1%−2% of the whole of neutrase solution used and the breakdown of casein in cheese due to nonencapsulated neutrase was 50% while, 40% in the case of encapsulated neutrase. Although, liposome retention rate in cheese curd was found to be 17%, which was significantly low, the slow release of liposome encapsulated neutrase led to an increase in the ripening period. Hence, the enrichment of cheese using liposomes can prevent potential barriers in the acceleration of dairy ripening (Law & King, 1985).

Several experiments were performed to accelerate cheese ripening, one of these procedures was performed by Alkhalaf et al., where the use of neutrase encapsulation in charged liposomes, on Saint-Paulin cheese milk was investigated. Three charged liposomes were prepared: (1) neutral, (2) positive, and (3) negative, and Neutrase was incorporated with each of these charged liposomes. Two assays were conducted to determine the encapsulation efficiency of each liposome: radioactivity and proteolytic activity. It was discovered that, the encapsulation efficiency of negatively charged liposomes was more than the positively charged or neutral liposomes; whereas, the liposome retention percentage of positively charged liposomes was higher (42%) than neutral (24%) and negatively charged liposomes (31%) in Saint-Paulin cheese. Furthermore, positively charged liposomes were found to be more stable at neutral pH, a lower

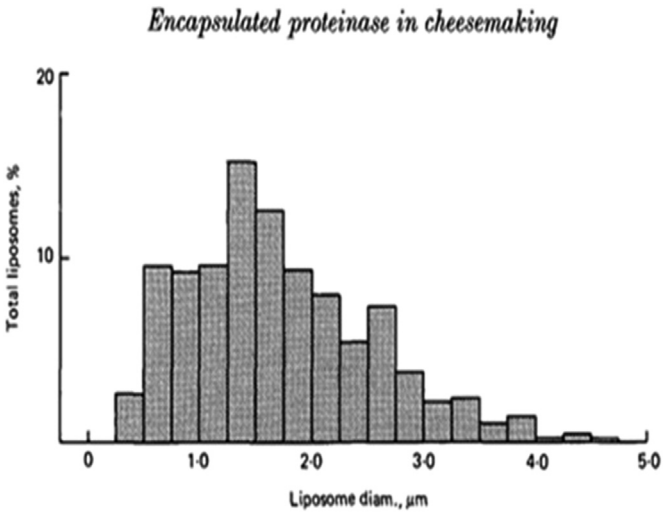

FIGURE 25.2 Size distribution of liposomes, estimated from negatively stained electron micrographs. *Reprinted with permission from Law, B. A. & King, J. S. (1985). Use of liposomes for proteinase addition to cheddar cheese.* Journal of Dairy Research, 52, *183−188.*

temperature (20°C) and low NaCl concentration. This experiment confirmed that the use of charged liposomes in the dairy industry can lead to novel developments in the manufacturing of cheeses (Alkhalaf et al., 1989).

25.2.1.3 Microcapsules

Kwak et al. developed another method to fortify cheddar cheese by microencapsulation of iron and L-ascorbic acid. Iron microcapsules of size 2−5 μm (using ferric ammonium sulfate) and L-ascorbic acid were coated with (polyglycerol monostearate) PGMS. The samples containing encapsulated and unencapsulated iron and L-ascorbic acid were prepared by adding microcapsules to cheddar cheese and stored for 7 months at 5°C. Concisely, it was reported that the encapsulation efficiency of iron and L-ascorbic acid microcapsules were recorded to be 72% and 94%, respectively. However, lipid oxidation was found to be slower in encapsulated iron and L-ascorbic acid samples than unencapsulated samples (TBA). It has to be noted that, even though sensory properties did not show any major differences, the production of neutral volatile flavor compounds (acetaldehyde) was higher in samples containing only iron (0.50−0.62 ppm) than samples containing L-ascorbic acid (0.26−0.35 ppm) at 7 months, respectively. Thus, it was concluded that microencapsulated iron and L-ascorbic acid yielded better results without any alteration in the quality (sensory properties) of cheddar cheese (Kwak et al., 2003).

Other researchers conducted a similar experiment, where vitamin C (Vit C) was used instead of L-ascorbic acid. Then, the microencapsulated iron and Vit C were used for the fortification of drinking yogurt. The yogurt samples were prepared by adding microcapsules of Vit C and iron (size of 2−5 μm and coated with PGMS); and unencapsulated Vit C and iron in yogurt and stored for 20 days (5−10−15−20) at 4°C. These researchers, reported the microencapsulation efficiency of iron and Vit C as 73% and 95%, respectively. Acidity increased throughout the storage period, whereas microbial growth and pH decreased within 20 days. Lipid oxidation (TBA) was comparatively slow in encapsulated iron, while there were no drastic changes in the sensory properties. Therefore, the overall result proved the importance of the encapsulation method in dairy processing (Kim et al., 2003).

25.2.2 Nanomaterials used for quality control

Quality of dairy products is termed as “optimum” when there are no unnecessary additives, bad odor, and free of unwanted chemicals, pathogens, and preservatives that lead to early microbial spoilage of the products. To overcome these issues, nanomaterials such as silver nanorods, liposomes, and nanocomposites have been incorporated to the dairy products.

25.2.2.1 Fe_3O_4 nanoparticles-carbon nanotubes interface

Before improving the quality, dairy products go through certain tests that estimate the quality, before being processed and reach the consumer. The industrially exploited chemical compound, hydrogen peroxide (H_2O_2), which was used to improve milk quality, was found to be harmful to human health when consumed at higher levels. Therefore, a catalase labeled biosensor (CAT/Fe_3O_4NPs-CNT/Au), made up of iron oxide nanoparticles (synthesized by coprecipitation of Fe^{2+} and Fe^{3+} aqueous solutions) integrated with carbon nanotubes consecutively, and gold electrode was introduced to detect the levels of H_2O_2. When this nanocomposite was used against raw milk containing H_2O_2, a higher detection range (1.2−21.6 μM), enhanced sensitivity (5.732×10^{-5} μAμM/cm^2) and decreased response time (less than 1 second) were reported. Along with these advances, detection limit of 3.7 nM and quantification limit of 12.2 nM were also noted. Upon studying the FE-TEM morphology images (Fig. 25.3), it was observed that the carbon nanotubes were surrounded by clustered Fe_3O_4 nanoparticles, giving catalase enzyme access to these nanomaterials and preventing alteration in their properties, thus yielding better results than the widely used standard biosensors (Thandavan et al., 2015).

25.2.2.2 Silver and gold nanorods

Now, quality is the most crucial aspect for health-conscious consumers, significant tests using silver nanorods (synthesized with the help of dairy wastewater) were performed to remove pathogens and maintaining the quality of milk. Analysis of the obtained silver nanorods using XRD, TEM, and energy dispersive X-ray analysis (EDAX) revealed, the face-centered cubic structure of a silver nanorod, non-agglomerated silver nanorods and the presence of only the Ag element in the obtained silver nanorods, respectively. Subsequently, diluted raw milk samples, containing different concentrations of silver nanorods, were put through MBRT (methylene blue reduction test) to assess the quality of milk. After that, standard plate count (SPC) and coliform methods were used to determine the microbial population. The obtained results showed a reduction in microbial growth from 2×10^7 to 29×10^5 CFU/mL and 23×10^5 to 7×10^5

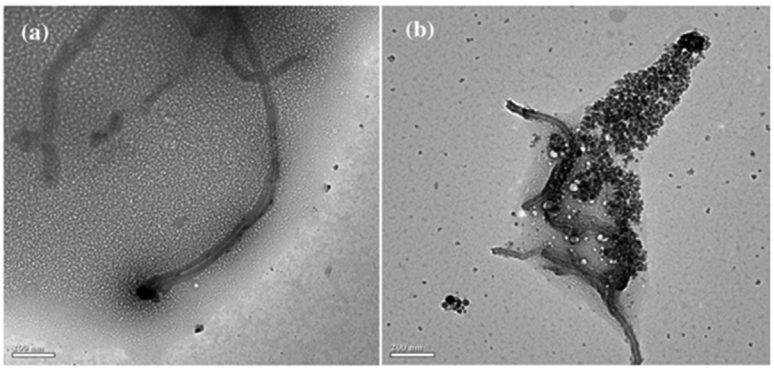

FIGURE 25.3 FE-TEM images of the (A) CNT and (B) CAT/CNT−Fe_3O_4 nanocomposite. *Reprinted with permission from Thandavan, K., Gandhi, S., Nesakumar, N., Sethuraman, S., Rayappan, J. B. B., & Krishnan, U. M. (2015). Hydrogen peroxide biosensor utilizing a hybrid nano-interface of iron oxide nanoparticles and carbon nanotubes to assess the quality of milk.* Sensors and Actuators, B: Chemical*, 215, 166−173.*

CFU/mL after 5 and 45 min, respectively. Furthermore, after 5 min, there was a gradual decrease in the concentration of silver nanoparticles, thereby increasing the microbial population due to the lack of reaction between AgNPs and microbes. Therefore, this study shows that the quality of raw milk can be maintained using silver nanorods without causing any changes in its nutritional value and thus extending its shelf life (Sivakumar et al., 2013).

Certain experiments were also performed, where *Escherichia coli* (used as an indicator bacteria) was detected through amplified electrochemical immunoassay to assess the quality of a dairy product. Gold nanorods (produced by seed-mediated synthesis) of size 10 nm in diameter and 40 nm in length (TEM morphology), were used to prepare gold nanorod-based labels ($_d$Ab−AuNR−FCA). Apart from the labels, a capture antibody ($_c$Ab)-based immunosensor was exercised to apprehend *E. coli*. The process started with an introduction of ($_c$Ab)-based immunosensor to a gold electrode, which was incubated along with *E. coli* for the bacteria to adhere to the capture antibody. Successively, ($_d$Ab−AuNR−FCA) label was added to the ferrocene monocarboxylic acid (FCA) to immobilize and bind to the *E. coli*. Upon immobilization, FCA was detected by differential pulse voltammetry (DPV) and the concentration of *E. coli* (varying from 1.0×10^2 to 5.0×10^4 CFU/mL) was confirmed, based on the resultant peak of DPV, estimating the quality of a dairy product. Therefore, the recoveries of standard additions ranged from 95.1% to 106%, proving electrochemical immunoassay using ($_d$Ab−AuNR−FCA), a more specific and stable label than conventional immunoassay methods (Zhang et al., 2015).

25.2.2.3 Nanoemulsified essential oils

Another experiment was conducted to preserve milk quality, where *Thymus capitatus*'s essential oil (extracted by hydro-distillation of dried ariel parts of *T. capitatus*) was encapsulated in a nanoemulsion and released into deliberately contaminated milk. The encapsulation process was performed by a high-pressure micro-fluidizer, followed by contamination of milk using gram-positive bacteria . For this experiment, three samples were considered, containing: (1) CM − contaminated milk, (2) CMEO − essential oil and contaminated milk, and (3) CMNE − nano-encapsulated essential oil and contaminated milk. After 24 hours of incubation, the results showed a reduction of 95% of the bacterial population in samples of CMEO and CMNE. However, only 37% protein reduction content was observed in the CMNE sample, while CM showed 50%. Furthermore, lipid oxidation was constrained by nano-emulsified essential oils, preventing the deterioration of its nutritional value and shelf life of semi-skimmed UHT milk. Comprehensively, essential oil encapsulated in water nanoemulsion showed better results than essential oil alone (ben Jemaa et al., 2017).

25.2.2.4 Nanopowder

The quality of dairy products can be preserved by maintaining the physicochemical properties of that product. Since health-conscious consumers prefer low cholesterol dairy products, Seo et al. performed an experiment to manufacture nano-powdered chitosan (NPC) to improve low cholesterol in yogurt. Therefore, β-cyclodextrin was used to remove cholesterol, consecutively, and samples with different concentrations of NPC (0.1, 0.3, 0.5, 0.7% wt./vol.) were added to cholesterol-removed yogurt and were put under observation for 20 days. It was reported that upon adding NPC, 93.5% cholesterol was reduced, while in its absence, 93.1% was reduced. Furthermore, the viscosity increased for about 15 days, and the color change was noticed on the 20th day of the experiment. As the concentration of NPC was increased, the lactic acid bacteria population decreased. In the case of pH, when NPC was supplemented, at day zero, pH raised from 4.33 to 4.47, and no major pH changes were observed for the next 20 days. However, when NPC was not supplemented, extreme changes in pH were observed and leading to noticeable spoilage of the yogurt.

Comprehensively, it was concluded that the use of optimum concentrations of NPC (0.3−0.5% wt./vol.) produced better results, proving the benefits of using nanomaterials in dairy industries (Seo et al., 2009).

Similar to the previous experiment, nano-powdered peanut sprout (NPPS) was compared to powdered peanut sprout (PPS), for preservation of dairy products in terms of physicochemical and sensory properties. The NPPS (300−350 nm) and PPS were synthesized by ground milling methods, subsequently added to yogurt at different concentrations (0.05%, 0.10%, 0.15%, and 0.20%) and stored for 16 days at 4°C. It was observed that, the viscosity increased with an increment in the concentration of NPPS, and the overall viscosity of NPPS was higher than PPS. The pH on day 0 was normal in NPPS-added samples and higher in PPS-added samples, even though there was a drastic decrement in the pH of NPPS-added yogurt on day 4, but as the storage time increased (day 16), the pH decreased gradually (4.45−4.25). However, there was no notable change in the color concerning the storage period, but the microbial growth slowly decreased (by day 16) with an increase in the concentration of NPPS. As in the case of the sensory properties, with an increment in the concentration of NPPS, the peanut flavor increased, the whey-off score increased, and the yogurt started turning yellow in color. Therefore, the reported data, confirmed the advantage of using NNPS over PPS, eventually proving the utilization of nanotechnology as the right choice in the dairy sectors (Ahn et al., 2012).

25.2.2.5 Liposomes

With an increase in the number of lactose-intolerant populations, the need for the digestion of lactose in milk before consumption has increased. Rodríguez-Nogales and Lopez designed an experiment to hydrolyze lactose using liposome encapsulated β-galactosidase in milk. Small unilamellar vesicles (SUV) were formed by using the DRV (prepared using cholesterol-Ch and Phosphatidylcholine-PC) method followed by encapsulation of β-galactosidase. It has to be noted that, the screening of influential experimental factors was conducted by using fraction factorial design. The optimum results showed that, an increase in encapsulation efficiency of liposomes enzyme: lipid ratio should be higher, Ch:PC ratio should be lower and, lastly, a bath-type sonicator was required. Although pH (pH 6) did not play any key role throughout this experiment, a second fraction factorial design was observed in which enzyme:lipid (13.76) and Ch:PC (0.53) were studied together as a combined effect. In the end, encapsulation efficiency of 28% was achieved, which was sufficient in delivering β-galactosidase in milk for hydrolysis of lactose (Rodríguez-Nogales & López, 2006).

Kim et al., reported a similar experiment to hydrolyze lactose in milk using β-galactosidase, dried liposomes (dehydration−rehydration vesicles method) mixed with trehalose to encapsulate β-galactosidase. Trehalose was used to increase the encapsulation efficiency, as it stabilizes the liposomal membrane and prevents leakage of β-galactosidase. The encapsulation efficiency of liposomes with trehalose was found to be four times better than the encapsulation efficiency without trehalose. The dried liposomes were reconstituted by rehydration; the stability of liposomes was studied by considering two factors: (1) reconstitution of liposomes (concentration of phosphatidylcholine), and (2) β-galactosidase retention. Upon investigation, it was discovered that, liposomes when stored at low temperature 4°C and 17°C for 60 days, 98.3% and 92.9% of dried liposomes were reconstituted, whereas the activity of β-galactosidase was about 87% and decreased to 70% at 37°C. The lysis or breakdown of the liposomes was found to be taking place in the intestine due to the presence of bile salts. Although they were lysed in the duodenum of the small intestine, it was observed that the liposomes were barely lysed in the stomach. Considering all the aspects, it was proved that dried liposomes with trehalose had superior effects over lactose than MLV liposomes (Kim et al., 1999).

25.2.3 Nanomaterials used as antimicrobial agents

The use of nanoparticles to increase or incorporate prolonged antimicrobial activity not only helps in improving the quality of dairy products but also increases the shelf life and enrichment of the products.

25.2.3.1 Nanolaminate coating

Mesophiles and psychrophiles are the most common bacteria found in cheese. To prevent the spoilage of "coalho" cheese due to microbes and increase its shelf-life, Souza et al., performed an experiment using nanolaminate coating by the layer-by-layer deposition method. The coating materials (alginate and lysozyme) were selected based on (1) electrostatic force (for conjugation between two layers), (2) antimicrobial properties, (3) gas barrier properties, and (4) antioxidant properties. Characterization using zeta-potential (alginate -60.97 ± 2.74 mV at pH 7.0, lysozyme $+29.27 \pm 3.18$ mV at pH 3.8), FTIR (confirmed the presence of amino groups on aminolysed/charged polyethylene terephthalate), and Scanning Electron Microscopy (SEM) (showed the morphology of the five-alternate alginate/lysozyme nanolayers) was also conducted. Four samples were prepared using coated and uncoated cheese with mesophilic

and psychotropic micro-organisms in each, separately. Results revealed a decrease in the microbial count in samples containing coated cheese (6.05 ± 0.05 to 7.7 ± 0.07 log CFU/g- mesophiles and 5.34 ± 0.04 to 7.47 ± 0.02 log CFU/g-psychrophiles) and an increase in the uncoated samples (6.2 ± 0.06 to 8.16 ± 0.01 log CFU/g- mesophiles and 5.68 ± 0.17 to 7.72 ± 0.05 log CFU/g-psychrophiles) (Medeiros et al., 2014).

25.2.3.2 Nanovesicle and liposomes

Nisin, manufactured using strains of *Lactococcus lactis*, is a peptide used as an antimicrobial agent in food industries. This experiment mainly deals with the effect of nisin encapsulated liposomes on *Listeria monocytogenes* present in milk. *Listeria monocytogenes* is a bacterium primarily found in dairy products due to its high tolerance to low temperatures. In order to eliminate bacterial growth, Malheiros and co-workers produced nanovesicles (thin-film hydration method) of 140 nm average diameter. Bacterial culture was prepared using BHI agar plates and was suspended in milk samples with an initial microbial count of 4 log CFU/mL. Encapsulated and unencapsulated nisin were added to the BHI agar plates and kept for incubation at 37°C for 24 h. Consecutively, the incubated encapsulated and unencapsulated nisin were added to the milk samples previously prepared to check antimicrobial activity. It was discovered that, the encapsulation efficiency of nisin-loaded liposomes was 94%. However, at 30°C, the antimicrobial activity of unencapsulated nisin was higher than encapsulated nisin. But at low temperatures ($\sim$7°C), encapsulated and unencapsulated nisin together helped in the decrement of the microbial population (da Silva Malheiros et al., 2010).

When added directly, nisin Z (bacteriocin), due to its affinity towards fats and proteins, could not reach the bacterial cells. Hence, liposomes containing nisin Z were introduced by Laridi et al., which decreased the binding of nisin Z to fats and proteins. Liposomes (740 nm average diameter) were manufactured by mixing proliposomes such as Pro-LipoC, Pro-Lipo Duo, Pro-LipoS (25°C), and Pro-LipoH (65°C) and nisin Z. Encapsulation efficiency (EE) of liposomes considering different parameters such as proliposomes, pH levels, stability, cholesterol content, and nisin Z concentration was observed (using competitive enzyme immunoassay). Overall, it was noted that the EE of liposome H was the highest (34.6%) while the lowest was found in liposome C (9.5%). Furthermore, increments in cholesterol content led to a decrement in EE; whereas, increments in nisin Z concentration (0.1−5 mg/mL) led to an increment in EE (34.6%−47%). Low pH was found to be suitable for EE of liposomes H and S and not for liposomes C and Duo, also TEM revealed intact vesicles with the immune signal. The acidic environment around the liposomes and increased fat content led to an improvement in the stability of liposomes thus increasing the bioavailability of nisin Z to the bacterial cells and increasing the shelf-life of milk (Laridi et al., 2003).

Similarly, liposomes containing nisin Z was used to study antimicrobial activity against *Listeria innocua, Lactococcus* spp. and *Lactobacillus casei* subsp. *casei* in cheddar cheese. Proliposomes were used to produce nisin (*Lactococcus lactis* subsp. *lactis* biovar diacetylactis UL 719-nisin producing strain) encapsulated liposomes and immune-TEM was used to check size distribution. Samples were prepared using nisin encapsulated liposomes and nisin producing strains individually. It was observed, although nisin encapsulated liposomes were successful in preventing *L. innocua* growth, they were not able to completely inhibit the growth of *Lactococcus* and *L. casei*. Through immune-TEM and gold-labeled anti-nisin Z monoclonal antibodies, it was observed that nisin-loaded liposomes were generally found at sites where fat or casein were present, while in the case of nisin-producing strains, they were scattered all over the cheese matrix (yet accumulating near fat-containing areas with the storage period). With this experiment, it was proved that liposomes are superior to any other modes of antimicrobial activity (Benech et al., 2002).

25.2.3.3 Nanohydrogels

Cheese contamination has been a critical issue since the early century. Therefore, to overcome this barrier, several experiments were performed, one of which was the use of thermosensitive nanohydrogel coating containing pimaricin (NP) on Arzúa-Ulloa DOP cheeses to prevent spoilage. Acrylic acid copolymerized PNIPA nanohydrogel coating containing pimaricin was prepared; simultaneously, Arzúa-Ulloa DOP cheese was deliberately contaminated using *S. cerevisiae*. Two bioassays were performed on cheese samples by (1) BIO1−natural contamination, and (2) BIO2−artificial contamination. It was reported that cheeses coated with NP showed less weight-loss than cheese coated without NP (after 30 days), in both the assays. Yellowness in color started appearing in both assays, while there was no significant effect of pH on samples containing NPS. It was also observed that the microbial count decreased without causing any disturbance to the natural ripening process of cheeses. A new qPCR method was introduced as a replacement to SPC, thereby studying the fungal counts in cheeses. It was noted that, qPCR was faster, sensitive, easy to handle, and more reliable than SCP (Standard plate count). Hence, nanohydrogels play an important role in reducing fungal growth and microbial population in cheese (Fuciños et al., 2017).

Since metal nanoparticles are excessively used in dairy industries for their antimicrobial properties, the following experiment investigated the use of agar hydrogel filled with silver montmorillonite nanoparticles (Ag-MMT NPs) for increasing the shelf life of Fiord latte cheese. The Ag-MMT NPs (made using ion-exchange reaction) were tested in three different concentrations (10, 15 and 20 mg) for 7 days. It was observed that when samples containing Ag-MMT NPs were used, the microbial count decreased to such an extent that the storage time could be increased, while samples not containing any NPs showed an increase in the microbial count. Since the sensory score was based on the sensory properties (color, texture and odor) analysis tests proved that Ag-MMT NPs gave better results, hence the sensory score was higher. Also, yeast counts of Ag-MMT NPs-loaded agar hydrogel samples were decreasing during the storage time. Keeping all these aspects into account, Incoronato et al., stated that silver nanoparticles play an important role in inhibiting bacterial growth and increasing the shelf-life of dairy products (Incoronato et al., 2011).

25.2.3.4 Nanocomposite coatings embedded with copper nanoparticles

Polylactic acid (PLA) films are used widely as a barrier against microorganisms that are responsible for the spoilage of cheese. Conte and his collaborators designed an experiment involving the use of nanocomposite coating with copper nanoparticles (CuNPs) embedded with sustainable packaging of Fiord latte cheese. The CuNPs (synthesized by picosecond-pulsed laser ablation) were embedded in an active PLA film through two methods: (1) mixing CuNPs and PLA (Cu_PLA_A), and (2) PLA in acetone water during laser ablation of CuNPs (Cu_PLA_B). Upon observing UV-vis and XPS readings, plasmon bands in all samples (around 580 nm) and the presence of Cu, O and C (content) on the surface of the coating were noted. Results revealed that, Cu_PLA_A_2 released copper nanoparticles rapidly than Cu_PLA_B_2 and, Cu_PLA_A_1 surpassed Cu_PLA_B_1 in preventing microbial activity, in vitro and in vivo tests (Fig. 25.4). It has to be noted that, the release rate of Cu^+ ions influenced the pH and microbial count. Therefore, this experiment showed a detailed idea of the advantages of the application of nanomaterials and sustainable packaging in dairy products (Conte et al., 2013).

25.2.4 Nanoparticles used as delivery agents

25.2.4.1 Emulsion and micelles

Helene et al. applied water-in-oil-in-water (W/O/W) double emulsion as a carrier of vitamin B12 for cheese fortification. The use of W/O/W was encouraged as it provided a protective barrier against gastric juices and improves retention in cheese. First W1/O primary emulsion was prepared by adding water phase (W1) to oil (O), then adding vitamin B12 to the internal aqueous phase at 0.2% (w/v). For the next phase, 0.35 mass fraction (mf) of W1/O was added to 0.65 mf of W2 and stored at 40°C prior to analysis. For the external aqueous phase, skim milk and NaCN dispersion were considered. The coalescence index was measured which showed a significant increase in the size of the emulsion during storage. Laser diffraction was used to determine size of emulsion. Calculation of encapsulation efficiency showed a 96% increase for vitamin B12 encapsulation, skim milk or NaCN had no significant effect in encapsulation efficiency. To check the stability of emulsion, a NaCN stabilized emulsion was exposed to a gastric environment alongside a

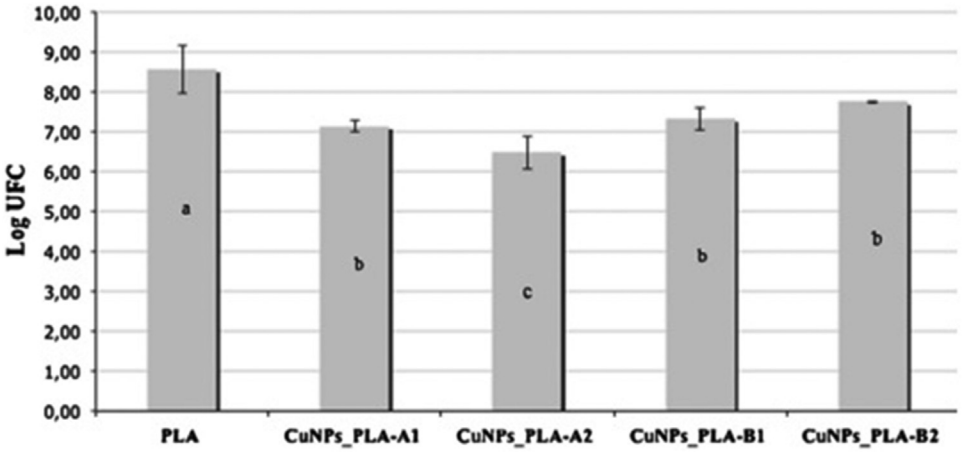

FIGURE 25.4 Results of control and copper−PLA films by in vitro test. Statistically significant differences (P .05) between samples are shown by different letters. *PLA*, Polylactic acid. *Reprinted with permission from Conte, A., Longano, D., Costa, C., Ditaranto, N., Ancona, A., Cioffi, N., Scrocco, C., Sabbatini, L., Contò, F., & del Nobile, M. A. (2013). A novel preservation technique applied to fiordilatte cheese.* Innovative Food Science and Emerging Technologies, 19, *158−165.*

control with no encapsulation. Results showed the percentage of vitamin B12 released from double emulsions was less than 4.4% after 120 min of gastric digestion (Giroux et al., 2013).

In another study, Michal et al. used casein micelles (CM) as a carrier to facilitate the delivery of vitamin D (VD). For this experiment, aggregates of CM and VD were formed. This was achieved by adding VD, pre-dissolved in ethanol, to caseinate solution leading to VD self-assembly. This process was interfered by adsorption of the casein onto the VD aggregates, stopping their growth and forming stable nanoaggregates (rCM). This was followed by the addition of citrate, phosphate and calcium (CCP) to induce CM reformation. To check if calcium was contributing to micelle formation, EDTA was added to VD3-rCM. The EDTA dissociates casein and vitamin by chelating calcium; results showed that with an increasing EDTA concentration, the turbidity gradually decreased, indicating micelle disintegration. To evaluate rCM protection of VD3, four systems were considered (unhomogenized VD3-rCM, homogenized VD3-rCM suspension, VD3-rCM in water and Tween-80 emulsion). After heating, VD3 concentration in both water and Tween-80 was lowered; whereas, in both unhomogenized and homogenized VD3-rCM suspensions, heating did not cause any significant reduction in vitamin concentration. In cold storage, both homogenized and unhomogenized VD3-rCM suspensions were more stable as compared to VD3 in water and Tween-80 emulsion. Hence, it was proved that encapsulation provided no vitamin loss under both conditions, showing effective encapsulation efficiency (Haham et al., 2012).

Thom and Cornelius studied nanogel particles prepared by enzymatic cross-linking of casein micelles. Casein was capable of cross linking themselves inside casein micelle in the presence of TGase creating nanogels particles. Furthermore, MCP can also be easily removed from nanogels without affecting the structure. For this study, serum protein-free milk was considered. After the preparation of casein micelles suspension from the milk sample, TGase was added to it and SDS analysis was conducted, thus confirming that TGase had no influence on both cross linking and size of micelles. When zeta potential was calculated for normal casein, the results were comparable to casein nanogel, indicating that the crosslinking process was insignificant on the zeta potential of the micelles. It was also seen that, zeta potential was not affected by micellar calcium phosphate (MCP). However, after changing MCP concentration from 0% to 150%, the colloidal stability of micelles was affected. Therefore, it was mentioned that adding TGase to casein micelles leads to well defined and stable nanogels. Upon performing heat and acid induced coagulation, it was seen that casein micelles were more stable to heat induced coagulation than acid induced. After running all these tests, it was proven that casein micelles were stable and could be used for applications such as estimation of mineral changes in milk samples or for transporting minerals (Huppertz & de Kruif, 2008).

25.2.4.2 Liposomes

Shuqin et al. reported on the preparation of ferrous sulfate liposomes with high encapsulation efficiency and physical stability. For doing so, numerous tests were performed to improve the efficiency of liposome. The encapsulation efficiency (EE) was studied by encapsulating ferrous ion solutions in different hydrating media (deionized water, citric acid−Na_2HPO_4 buffer solution and citric acid−sodium citrate buffer solution). Four different methods were considered for preparation of ferrous sulfate liposomes in order to compare their EE. These methods were reverse-phase evaporation (REV), thin-film sonication (TFS), Thin film hydration (TFH) and freeze-thawing (FT) After the liposomes were prepared via these methods, they were subjected to bathophenanthroline colorimetry for calculation of EE. The highest EE was found for REV and, hence, it was used for further investigation. Zeta potential was also calculated using the REV method and the results showed that incorporation of cholesterol in liposomes increased zeta potential at 25°C. Also, more concentration of cholesterol resulted in a high zeta potential (Fig. 25.5). The best hydrating media was

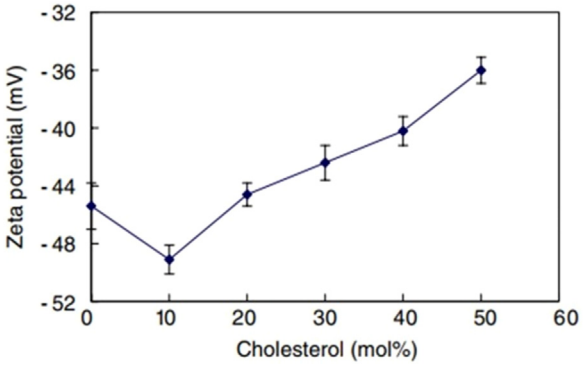

FIGURE 25.5 ϑ-Potential change of PC-liposomes with various amounts of incorporated cholesterol concentration. Conductivities are all 0.01 Ms/cm. *PC*, Phosphatidylcholine. *Reprinted with permission from Xia, S. & Xu, S., (2005). Ferrous sulfate liposomes: Preparation, stability and application in fluid milk.* Food Research International, *38, 289–296.*

found to be citric acid−Na_2HPO_4 buffer because citrate can chelate ferrous ions, which improves EE of iron in liposomes. Therefore, after the removal of free ferrous ions, liposomes were added to milk for visual observation. Results during week 1 storage showed that there were no changes in color or sensory factors of milk, and also there were no signs of precipitation or coagulation of liposomes. These results prove that ferrous sulfate liposomes can be applied in liquid milk samples for improving Fe ion stability (Xia & Xu, 2005).

Mashsid et al. evaluated the effect of flavourzyme (protease) loaded nanoliposome added to milk for accelerating the ripening of Iranian brined cheese. This research group also used whey and curd for the evaluation of liposomes. Firstly, liposomes were manufactured, then this liposomal flavourzyme was added to the milk at its renneting stage to check for acceleration of ripening cheese. Curd and drained off whey were also prepared in the laboratory and eventually added with flavourzyme. For observation, water soluble nitrogen (WSN) and non-protein nitrogen (NPN) were determined. The NPN was determined in the 12% TCA-soluble fraction, and expressed as a percentage of total nitrogen (TCA-SN/TN). The results for curd and cheese showed that pH of WSN/TN (%) and TCA-SN/TN (%) were not significantly influenced by the addition of flavourzyme; however, the TCA-SN of whey was significantly increased. It was also reported that the early proteolysis of casein was inhibited by flavourzyme during cheese production and also inhibited the pre-maturation of protein curd (Jahadi et al., 2015).

Laloy and collaborators also applied liposomes as delivery vehicles to deliver enzymes for cheese ripening. For this study, alpha-chymotrypsin was used and the free enzymes were separated from immobilized ones via centrifugation. Characterization was conducted by calculating immobilization efficiency (IE), proportion of immobilized enzyme at surface of the vesicles (IS), the proportion of immobilized enzyme entrapped inside liposomes (IEEL) and (EPL) i.e., amount of enzyme (mg) immobilized per 100 mg of lipids. The results showed that an IS of 37%, IE of 94%, EE of 63% and EPL ratio of 11% was achieved. Parameters influencing this study investigation were fat content and pH of cheese. The obtained results showed that enzymes release from liposomes occurred within 1 week of ripening; this might be due to an interaction between milk globules i.e., the presence of fat influences the early release. Although enzyme release started early, after 2 months of ripening proteolysis was 30% lower in liposome, indicating enzyme release inhibition (Laloy et al., 1998).

In another experiment, liposomes were used and the aim was to employ liposomes to accelerate the fat breakdown process during cheese ripening. For this study, two types of lipase (Palatase M and Lipase 50) were entrapped in liposomes. Encapsulation efficiencies (EE) were found to be 35.9% and 40.3% for Palatase M and Lipase 50, respectively. The experimental cheese was slightly higher in moisture content than the control cheese along with less protein and fat content. It has to be noted that, higher moisture content can be accounted for because of the phenomenon of water binding at the surface of liposomes. The experimental cheese also showed higher proteolysis along with increase in nitrogen fractions (TCA/SN, WSN) during ripening. Therefore, the addition of liposomes does not hinder the sensory factor of cheese when compared to the addition of free lipases (Kheadr et al., 2002).

Picon et al. used dehydration−rehydration liposomes to encapsulate neutral protease extracted from *Bacillus subtilis* (BSNP) and was used to pasteurize milk for accelerating manchego cheese ripening. After the preparation of liposomes, BSNP was added, then subjected to sonication, freeze drying and rehydration. Un-encapsulated and liposome-encapsulated (LE) proteinases were separated via centrifugation and were then stored below 4°C. To calculate the EE and activity of BSNP, liposomes were mixed with PBS and then disrupted via sonication. The results showed that after centrifugation, BSNP activity was 54.3%; whereas, after sonication, LE accounted for 21.2% of the initial BSNP activity; there was a loss of 24.5% BSNP activity during the encapsulation process. It was also observed that, LE BSNP added to milk accelerated the degradation of both *alphaS1* and beta-casein in milk, thereby indicating enhanced ripening of the cheese. Also, the addition of LE BSNP to cheese decreased its protein content, but showed no pH effects. Despite the remarkable increase in proteolysis, the bitterness ratio was low and there was no evidence of LE BSNP influence to the cheese milk (Picon et al., 1995).

25.2.5 Nanoparticles for detection

Dairy products are prone to spoilage due to changes in temperature or pH during storage or transport. Hence, there is a need to monitor dairy products by detecting pathogenic microbes or various substances that are toxic above a certain limit. However, the traditional detection methods do not offer rapid and efficient detection. Therefore, there has been development of novel techniques using nanoparticles which offer greater sensitivity and excellent reliability. Given below are studies that investigated the application of nanoparticles in detection of various diary product components.

25.2.5.1 Metal nanoparticles and quantum dots

There are various techniques devised to detect melamine content in milk. Determining melamine content is essential, because if it is ingested above safety limits it might result in the formation of cyanurate crystals in the kidneys. Therefore, Li et al. developed an effective colorimetric detection method for the determination of melamine content in milk samples with different concentrations of gold nanoparticles (Fig. 25.6). In this work, AuNPs were used as colorimetric probe material. The theory behind using AuNPs is that, melamine can easily bind to its surface through amine groups, leading to color change from red to blue, which indicates the presence of melamine. In order to test this theory, AuNPs were prepared using chloroauric acid and the second calorimetric analysis of milk for melamine was conducted. After centrifugation of sample, filtrate was added to AuNPs, absorption spectra and TEM were recorded. The experimental results showed that absorption ratio (A650/A520) increased with an increase in the melamine concentration, i.e., the more the AuNPs, the better the detection of melamine. Calorimetric detection photographs show that in the presence of melamine, the color of AgNPs change from red to blue, and with an increase in concentration of melamine (5×10^3 to 20×10^3 g/L), the color turned dark blue. The TEM results showed that, after the addition of melamine, aggregation of AuNPs occurred. The set detection limit was 0.4×10^{-3} g/L and the whole experiment duration was 12 min, proving that this is a rapid and effective method for melamine detection in milk samples (Li et al., 2010).

Lu et al. had also made a successful attempt in detecting melamine in milk via florescence enhancement of AuNPs. Melamine is a weak base, therefore, it gets protonated under acidic conditions (in this case cyanric acid). Therefore, the protonated melamine and AuNPs conjugate result in aggregation and color change. However, the initial approach was the same; after the synthesis of AuNPs, centrifugation was performed i.e., different concentration of melamine were added to diluted buffer solution and centrifuged. Zeta potential before and after the addition of NPs were −30.2 mV and −11.38 mV. It was also observed that, the florescence activity of AgNPs was decreased when concentration of melamine was above 8×10^{-8} M. The UV-Vis spectra was measured from 200 to 800 nm, in an excited wavelength of 252 nm, where NPs of 16 nm diameter can emit stable florescence at 370 nm. Hence, this method can detect trace levels of melamine in milk samples (Xiang et al., 2011).

In another work, a carboxyl-terminal poly (amidoamine) dendrimer-encapsulated AuNPs [PAMAM(Au)] based sensor was designed for immunoassay of *E. coli* in dairy products. This study was divided into three parts. The first part was the preparation of [PAMAM(Au)], the second part was the preparation of (dAb-CNT-HRP) nanoprobes by exploiting CNTs + Ab (antibodies) as carriers for loading HRP (horseradish peroxidase) and antibodies (Ab), and the third part was the formation of an immunosensor. For the third step, [PAMAM(Au)] was mixed with chitosan, then EDC and NHS were cast on [PAMAM(Au)/chit] for 30 min. This solution was then incubated with Ab which resulted in the formation of the immunosensor. Then, this sensor was incubated with *E. coli* at 37°C. The UV-Vis and TEM results showed that AuNPs were of 3 nm size and formed without aggregation. The TEM measurements also showed that after the addition of Ab, the size of NPs increased. The FTIR spectra confirmed Au encapsulation inside the PAMAM dendrimers. The DPV peak current showed that an increase in *E. coli* concentration increased the (dAb-CNT-HRP) nanoprobes captured on the electrode surface, proving these sensors to be promising. The advantage of using dendrimer-encapsulated metal NPs in this experiment was because both dendrimer and metal NPs have high density, good biocompatibility and conductivity (Zhang et al., 2016).

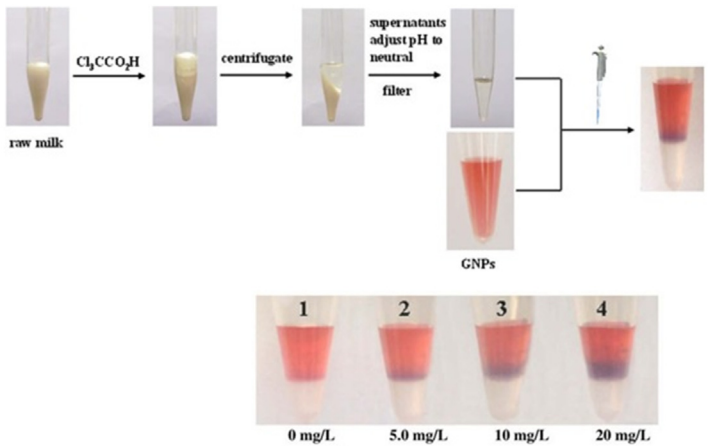

FIGURE 25.6 Schematic representation of the analytical process for detecting melamine in raw milk and photographs of GNPs solutions in the presence of melamine with different concentration levels. *Reprinted with permission from Li, L., Li, B., Cheng, D., & Mao, L., (2010). Visual detection of melamine in raw milk using gold nanoparticles as colorimetric probe.* Food Chemistry, *122(3), 895–900.*

Other collaborators used AgNPs for the detection of melamine in raw milk. The idea behind this investigation was, when melamine bond with AgNPs lead to color change, it can be observed with the naked eye or UV-Vis spectra. Typically, centrifugation is done for calorimetric analysis of milk; therefore, it was conducted after AgNPs were prepared. The obtained supernatant was then added to AgNPs and the absorption spectra was recorded which showed a peak at 402 nm, demonstrating that AgNPs were well dispersed. The TEM images of AgNPs justified that aggregation of NPs occurred due to the addition of melamine and, hence, the color change. This experiment was completed within 30 min and the detection limit of 0.29 mg/L were achieved (Ping et al., 2012).

A cost-effective method was developed for detection of milk spoilage in early stages. Ankita et al. used interactions between cysteine (-SH) and silver to prepare biofunctionalized AgNPs. The milk samples were taken from different parts of India. This experiment first required the formation of Ag NPs. Then, the addition of cysteine to these NPs with a ratio of 1:19 (mL/mL), keeping different concentrations of AgNPs (10^{-3} to 10^{-9} mol/L). The milk detection observations showed that after, 8 hours at 30°C, there was milk spoilage, indicating an increase in acidity, pH and LAB count, whereas, the COB test was positive. To check if lactic acid was detected by functionalized NPs, lactic acid (1−100 mL/L) was added to the NPs at room temperature. The results showed that, there was no color change observed when lactic acid was added to NPs without cysteine, indicating that cysteine was necessary for detection of milk spoilage. With time, the concentration of lactic acid increased in milk sample due to fermentation by LAB, which led to the aggregation of Ag + cysteine NPs and the color change (yellow to red). This color change can be observed with the naked eye, making this method convenient and rapid for detection of milk spoilage at a pre-spoilage point (Lakade et al., 2017).

In another similar experiment, melamine content in milk was detected by using CdTe quantum dots as probes. Formation of these quantum dots was conducted by capping them with thioglycolic acid (TGA), forming TGA-CdTe QDs. The only difference was the addition of cyanuric acid. The concept behind using quantum dots was because of the florescence reduction after the addition of melamine. As discussed above, the centrifugation of the milk sample was performed, the supernatant removed and in it quantum CdTe were added. The fluorescent emission spectra of TGA-CdTe QDs in the absence and presence of melamine were recorded. The results showed that there was no obvious change in the spectra for both cases. Also, with an increasing concentration of melamine, a red shift was observed, indicating change in surface and not in size of quantum dots. This experiment was completed within 30 min and the detection limit was 0.04 mg/L (Zhang et al., 2012).

25.2.5.2 MIP NPs and multi-walled carbon nano-tube

Detection of alpha-casein in milk using molecularly imprinted polymer nanoparticles (MIP NPs) by surface plasmon resonance (SPR) was conducted by Jon et al. The use of SPR was favored, since this method is cost-effective and offers good sensitivity to detect spiked casein levels in milk. This experiment required glass beads to be first functionalized with amines to attach protein (casein) as a template via amine coupling reaction. Then, MIP NPs were prepared using a solid phase-based synthesis approach. After that, the MIP NPs and beads were mixed inside a column already occupied with an unreactive monomer solution to facilitate mixing. The binding affinity of NPs were measured using a SPR biosensor by immobilizing them on the surface of an Au SPR chip and placing varying casein concentrations. This showed that nanoMIPs and protein had a 1:1 binding stoichiometry. The MIP NPs offered quantitative detection of casein with a detection limit of 0.127 ppm (Ashley et al., 2018).

Yogurt and cheese are crucial dairy products, which were used to determine natamycin by using on screen-printed carbon electrode (SPCE). The MWCNTs-Pt-doped CdS nanocomposite were firstly prepared by keeping different ratios of Pt-doped CdS and MWCNT. Then, sulfuric acid and natamycin were transferred to SPCE cells. After conducting x-ray diffraction analysis, hexagonal and cubic NPs were observed and no peaks of Pt were observed, indicating that Pt were completely embedded into CdS. It was also observed that the Pt-doped CdS ratio was proportionally increasing with the response of the electrode. The voltammograms displayed electrochemical behavior of natamycin on both modified and unmodified SPCE and showed an increase in oxidation peak of natamycin for MWCNTs modified SPCE, indicating their better sensitivity for natamycin. Also, to check its stability, the sensor was subjected to the experiment every 30 min and less than 10% reduction in natamycin content was observed. Therefore, this sensor proves to be very sensitive and easy to fabricate for the determining natamycin in yogurt and cheese samples (Yousefi et al., 2018).

25.2.6 Nanoparticles applied for packaging

Nanotechnology has enabled the development of a superior barrier in packaging material along with enhancing mechanical properties. Conventional nanomaterials were formed by combining synthetic polymers with inorganic solids. These

were not eco-friendly and did not provide long term solutions for packaging. Hence, nanotech was incorporated to increase the shelf life of packed products.

25.2.6.1 Nanoparticles

Considering bio-nanocomposites, an experiment was conducted on soft cheese for its packaging material. For karish cheese, with a short shelf life, it was very prone to pathogenic attacks. Therefore, some researchers combined chitosan with polyvinyl alcohol (PVA) and titanium oxide (TiO_2-NPs) nanoparticles. After the preparation of TiO_2-NPs, a polymer of chitosan and PVA were prepared in the ratio 30:70 (v/v). Then 0.5% glycerol was added to CS/PVA solution. The test was performed by adding different concentrations of TiO_2-NPs (1%, 2%, 3% w/v) to the CS/PVA/Gy/TiO_2 bio-nanocomposites solution. This suspension was directly coated onto karish cheese. These two groups were created, the first group without coating, 2nd group coated with CS/PVA/Gy suspension and the 3rd, 4th, 5th groups with CS/PVA/Gy suspensions containing 1%, 2%, and 3% of TiO_2-NPs, respectively. These samples were then stored in cold storage for 25 days. The XRD, SEM and TEM images showed that TiO_2-NPs were evenly distributed within the polymer. The water vapor transmission rate (WVTR) showed that composite with 3% TiO_2-NPs improves the water vapor barrier by 63%. Mechanical properties increased with the addition of nanoparticles; hence, 3% TiO_2-NPs show the best mechanical strength for packaging. Antimicrobial tests indicated that the nanocomposites show good inhibitory activity against bacterial species. The nitrogen content in uncoated cheese increased during the storage period. After all these evaluations, the highest concentration of TiO_2-NPs showed the best result in terms of shelf life and quality (Youssef et al., 2018).

In another experiment , a film of high-density polyethylene (HDPE) or calcium carbonate ($CaCO_3$) containing TiO_2 (1% w/w) was prepared for the packaging of short-ripened stracchino cheese. The properties of this film were evaluated by UV-Vis spectroscopy, gas chromatography, and pH meter, checking surface wettability by goniometer and an innovative approach of conducting photocatalytic activity on TiO_2. A container was prepared by using the film along with two pure polymers (PE and PS). The stracchino cheese sample was placed in the container for 8 hours at 4°C in a refrigerator, the light source was four warm white vertical lights. Then, the samples were stored in the dark at 4°C for 28 days. The UV-Vis spectroscopy showed that TiO_2 were homogenously distributed onto the film. Also, HDPE + $CaCO_3$ + TiO_2 film had a positive effect by showing a decrease in the degradation rate of cheese. When irradiated, TiO_2 promoted the release of CO_2, but its release was not significant. Also, on the basis of the results, the inhibitory activity of the film was due to the production of oxygen and not CO_2. Thus, this film is a cost effective, highly shapeable photoactive material that can be well applied in diary product packaging (Gumiero et al., 2013).

Mastromatteo et al. (2015) conducted an experiment to evaluate the effect of AgNPs combined with MAP (50% CO_2 and 50% N_2) on fior di latte cheese to prolong its shelf life and packaging. For this investigation, a cheese sample is dipped into sodium alginate solution then in calcium chloride (5% w/v) for 1 min. Furthermore, there was an active coating prepared by dissolving sodium alginic acid (2% w/v) and 0.25 mg/mL of AgNPs in water. Then the coated samples were placed in two containers, with and without coating, and MAP (modified atmospheric packaging). Analysis done by Marianna include antimicrobial check, shelf life and sensory analysis. Results show that there was a delayed microbial growth when packed with coating and MAP; moreover, Ag NPs slowed exponential growth for 10 days. Whereas, without MAP the threshold was obtained at 7 days. There was an increase in the microbial count in storage with and without MAP coating, but MAP packaging showed less increase in total viable count (TVC) (6 log CFU/g for 9 days of storage and 7 log CFU/g at 13 days), comparatively. The sensory analysis results showed that there was a need for coating on cheese before packing, otherwise the sample developed a yellow color. Although, the best results were shown by coating and not by MAP, MAP improved the overall quality of coated cheese (Mastromatteo et al., 2015).

Other researchers performed experiments that aimed at combining the effects of zeolite-X (ZX) and zeolite-X loaded with AgNPs (ZX-AgNPs), to form an effective packaging that can be applied to increase the shelf life of milk. After the formation of films, TEM analysis was performed, which showed similar size nanocrystals of both ZX and ZX-AgNPs. After the formation of films, the samples were divided into five groups, (1) control (free of ZX and ZX-AgNPs), (2) milk packaged with 0.5% ZX, (3) package with 1% ZX, (4) package with 0.5% ZX-AgNPs, and group (5) package with 1% ZX-AgNPs. These groups were injected into the blood of rats used for the experiment and the changes observed in rat organs were recorded. After monitoring all the groups and performing variety of tests, it was discovered that groups 4 and 5 showed the best results with no damage to organ cells. Therefore, these samples were considered for further evaluation. It was also noticed that there was migration of AgNPs from the packaging into the milk sample; therefore, ICP-AES was conducted, indicating the amount of Ag ions migration from 1% ZX-AgNPs films. It was

reported that there was more than 0.5% ZX-AgNPs that migrated to the milk solution. Therefore, all these data indicated that, due to the little toxic effect shown by 1% ZX-AgNPs, the best film to be used for packaging was 0.5% ZX-AgNPs (Elsherif et al., 2020).

25.2.6.2 Nanocomposite

In another experiment, nanocomposites of chitosan, cellulose, and nisin were used in the packaging of UF (ultra-filter) cheese. The developed films (both CC [cellulose chitosan] and NICC [Nissin-incorporated cellulose chitosan]) were analyzed to access swelling and water solubility percentage, and it was observed that the addition of nisin did not show any significant change in water content. The water uptake was important as it allowed the film to release antimicrobial agents for a long period of time. It was also observed that, during the first 4 hours of packaging, the film showed a considerable increase in swelling. To characterize ZnO nanostructures in the chitosan matrix, SEM and EDS were conducted. FESEM (field emission scanning electron microscope) showed the diameter of film to be of 36 micrometers and proves the presence of ZnO in the matrix, indicating its successful incorporation (Fig. 25.7). NICC films showed antimicrobial behavior against *L. monocytogenes*; according to the results 500 and 1000 ppm nisin had inhibition zones having 23 and 26 mm diameters. This proves chitosan to be good biopolymer to support nisin. Regardless of the concentration of nisin, its release in the sample takes at least 6 days. Therefore, for prolonged release of nisin, its entrapment in CC film was necessary, which was effectively conducted by the sol-gel method as shown in this experiment (Divsalar et al., 2018).

In another experiment, CS/PV/TiO_2 nanocomposite films were characterized (SEM, FTIR (Fourier transform Infrared Spectroscopy), XRD and TEM) and used as packaging material for soft white cheese. The result from XRD showed that nanocomposites were organized in a PVA semi crystalline structure. The SEM images showed that TiO_2 NPs were spherical shaped. It was noted that when the concentration of TiO_2 was low (2%), there were no CS/PVA

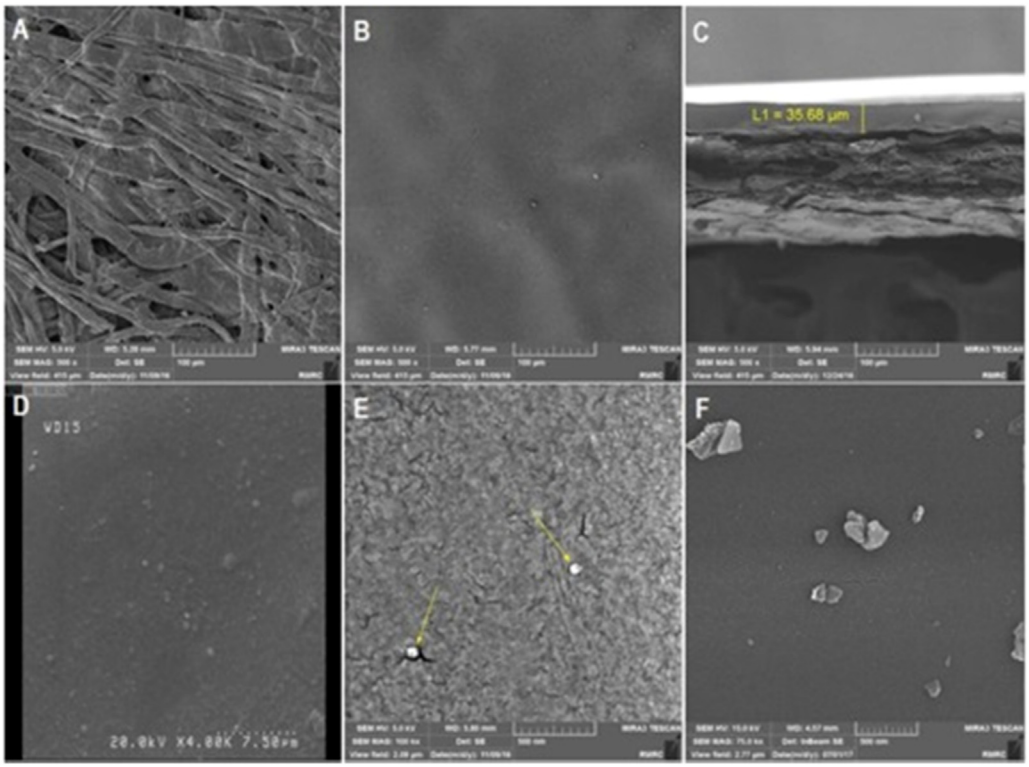

FIGURE 25.7 FESEM micrographs of cellulose films: (A) uncoated (magnification: 500×), (B) cellulose coated with chitosan (magnification: 1000×), (C): the cross section of film (magnification: 500×) (D), cellulose coated with chitosan-ZnO nanocomposite (3 wt.% magnification 20,000×), (E) cellulose coated with chitosan-ZnO nanocomposite (3 wt.% magnification 100,000×) and (F) cellulose coated with chitosan-ZnO nanocomposite after addition of 500 ppm Nisin (magnification 75,000×). *FESEM*, field emission scanning electron microscope. *Reprinted with permission from Divsalar, E., Tajik, H., Moradi, M., Forough, M., Lotfi, M., & Kuswandi, B., (2018). Characterization of cellulosic paper coated with chitosan-zinc oxide nanocomposite containing nisin and its application in packaging of UF cheese.* International Journal of Biological Macromolecules, 109, *1311–1318.*

TABLE 25.1 Type of nanomaterials used and their different application in dairy industry.

Type of nanomaterial	Applications	Characterization	Size	Dairy product used	References
Liposomes/dried/ multilamellar	Delivery	EE, zeta potential, Ultrasonication	-	Milk	Xia and Xu (2005)
		Encapsulation efficiency	179 nm	Cheese	Jahadi et al. (2015)
		-	-	Cheese	Laloy et al. (1998)
		-	-	Cheese	Kheadr et al. (2002)
		TEM, SEM, FTIR	-	Cheese	Picon et al. (1995)
	Enrichment	DLS	300–500 nm	Yogurt	Ghorbanzade et al. (2017)
		TEM	0.25–5 μm	Cheese	Law and King (1985)
		-	-	Cheese	Alkhalaf et al. (1989)
	Antimicrobial	Photo correlation spectroscopy, IR spectroscopy, TEM	740 nm	Milk	Laridi et al. (2003)
		Immune-TEM, gold-labeled antinisin Z monoclonal antibodies	80–120 nm	Cheese	Benech et al. (2002)
	Quality	-	-	Milk	Kim et al. (1999)
Au NPs	Detection	TEM, UV-Vis, absorption spectra, calorimetric detection	2.6 nm	Milk	Li et al. (2010)
		TEM, UV-Vis spectra	60 nm	Milk powder	Xiang et al. (2011)
	Quality	EIS, TEM, UV-vis NIR spectrometer	10 × 40 nm	Milk and yogurt	Zhang et al. (2015)
Nanocomposite	Detection	XRD, SEM, EIS, Voltammogram	20–30 nm	Yogurt drink and cheese	Yousefi et al. (2018)
		TEM, UV-Vis, FTIR, EIS	3 nm	Milk and yogurt	Zhang et al. (2016)
	Packaging	XRD, SEM, TEM, WVTR and mechanical strength	20–30 nm	Cheese (karish)	Youssef et al. (2018)
		FTIR, SEM, TEM	36 μm	Cheese	Divsalar et al. (2018)
		DPV, SEM, UV-Vis spectroscopy	-	Cheese	Gumiero et al. (2013)
		-	-	Cheese	Mastromatteo et al. (2015)
		SEM, TEM, XRD, FTIR	<100 nm	Milk	Elsherif et al. (2020)
		SEM, TEM, XRD, FTIR	<25 nm	Cheese	Youssef et al. (2015)

(*Continued*)

TABLE 25.1 (Continued)

Type of nanomaterial	Applications	Characterization	Size	Dairy product used	References
Ag NPs/nanorods	Antimicrobial		-	Cheese	Incoronato et al. (2011)
	Detection	TEM, UV-Vis spectra, calorimetric detection via centrifugation	-	Milk	Ping et al. (2012)
		UV-Vis, DLS, HPLC, Raman spec	52–72 nm	Milk	Lakade et al. (2017)
	Quality	TEM, UV-Vis spectrophotometer, EDAX	-	Milk	Sivakumar et al. (2013)
	Packaging	-	<100 nm	Milk	Elsherif et al. (2020)
Nanopowdered (chitosan, peanut sprout)	Quality	Particle size analyzer, SEM. SEM, particle size analyzer	562 nm	Yogurt	Seo et al. (2009)
		-	300–350 nm	Yogurt	Ahn et al. (2012)
Metal NPs (Ca, Fe, Zn, Cu, Fe_3O_4)	Enrichment	XRD, TEM, FTIR, TBA	50–80 nm	Yogurt	Santillán-Urquiza et al. (2017a)
	Antimicrobial	UV-vis spectroscopy, XPS, CAE, BE	580 nm	Cheese	Conte et al. (2013)
	Quality	Cyclic voltammograms, FE-TEM	-	Milk	Thandavan et al. (2015)
Nanoemulsion	Delivery	Coalescence index, laser diffraction, encapsulation efficiency (EE)	158–184 nm	Cheese	Giroux et al. (2013)
	Quality	Gas chromatography-mass spectroscopy analysis	110 nm	Milk	ben Jemaa et al. (2017)
Vesicles/ unilamellar	Quality	-	-	Milk	Rodríguez-Nogales and López (2006)
	Antimicrobial	Zeta-potential	140 nm	Milk	da Silva Malheiros et al. (2010)
Microcapsules	Enrichment	TBA	2–5 μm	Cheese	Kwak et al. (2003)
		TBA	2–5 μm	Drink yogurt	Kim et al. (2003)
Micelles/casein	Delivery	Encapsulation efficiency, dynamic light scattering, HPLC	50–500 nm	Milk	Haham et al. (2012)
		TEM, SEM, FTIR, SDS-PAGE	100 nm	Milk	Huppertz and de Kruif (2008)
Nanolaminate (Alginate/lysozyme)	Antimicrobial	SEM, zeta potential, FTIR, UV-Vis spectroscopy, OTR, contact angle	10 nm	Cheese	Medeiros et al. (2014)
Nanohydrogel	Antimicrobial	-	10–1000 nm	Cheese	Fuciños et al. (2017)

(*Continued*)

TABLE 25.1 (Continued)

Type of nanomaterial	Applications	Characterization	Size	Dairy product used	References
Quantum dots	Detection	Gradient method, florescence absorption spectra	2−2.8 nm	Milk	Zhang et al. (2012)
MIP NPs	Detection	TEM, DLS	228−263 nm	Milk	Ashley et al. (2018)

Note: *EE*- Encapsulation Efficiency, *TEM*- Transmission Electron micrscopy, *SEM*- Scanning Electron micrscopy, *FTIR* - Fourier Transform Infrared Spectroscopy, *DLS* - Dynamic Light Scattering, *UV-Vis* - Ultraviolet- Visible spectroscopy, *EIS* - Electrochemical Impedance Spectroscopy, *WVTR* - Water Vapor Transmission Rate, *DPV* - Differential Pulse Voltammetry, *HPLC* - High performance Liquid Chromatography, *EDAX* - Energy Dispersive X-Ray Analysis, *TBA* - Thiobarbituric acid test, *XPS* - X-ray Photoelectron spectra, *CAE* - Constant Analyzer Energy, *BE* - Binding Energy, *FE - TEM* - Field Emission Transmission Electron micrscopy, *SDS-PAGE* - sodium dodecyl sulphate−polyacrylamide gel electrophoresis, *OTR* - Oxygen Transmission Rate, *EIS* - Electrochemical impedance spectroscopy

nanocomposite, which demonstrated a homogenous surface. However, higher concentration (8%) showed heterogeneous surface, indicating aggregation of TiO_2 NPs. The TEM results also proved the same, the higher the concentration of TiO_2 the more the distribution. Mechanical strength of the film was measured via tensile strength (TS) and the TS increased with an increase in TiO_2 content (2%, 4% and 8%), which might be due to the fact that TiO_2 NPs were well dispersed in the film with increasing concentration. Therefore, the combination of TiO_2-NPs affected the crystallinity of CS/PVA, as revealed by XRD and FTIR results. The zone of inhibition was calculated for antimicrobial activity, showing that 8% TiO_2 had the best inhibitory effect. Alternatively, moisture content of cheese also increased with a storage period and from 2%−4% TiO_2 content; whereas, it decreased for 8% TiO_2 content. When the samples were tested for color change after 15 days of storage, the sample packed with nanocomposite films remained white in color; whereas, the sample with no packaging showed a yellow color. Also, the bacterial count reduced with the rising storage periods and completely vanished at the end, indicating that this film is a sustainable material for dairy packaging (Youssef et al., 2015).

25.3 Conclusion

Most of the experiments in this chapter hold a great significance towards the improvement of dairy products using nanomaterials. The use of nanomaterials, such as metal nanoparticles, liposomes, and microcapsules, in the enrichment of dairy products, produced a significant difference in the physicochemical and sensory properties, that is, color, texture, taste, odor, etc., of the dairy products (Table 25.1). Similarly, the quality of cheese, milk, and other dairy products were enhanced using nanointerface, nanorods, liposomes, nanoemulsified oils, and nanopowder. A comparison between nanopowdered chitosan/peanut sprout and powdered chitosan/peanut sprout was brought up to explain the importance of nano-sized particles and its developed properties. On the other hand, antimicrobial activity in the presence of nanomaterials, i.e., nanolaminate, nanohydrogel, nanocomposite, and nanoliposomes, was enhanced. In most of the cases mentioned, the microbial activity reduced by 2.0 log CFU/g or 2.0 log/g; this resulted in increased shelf life and improved quality of the dairy products. Another method used to improve the quality of dairy products was delivering nutrients using liposomes, micelles, and emulsions as delivery agents. It was observed that in the physicochemical and sensory analysis, after using nanomaterials in delivery, along with quality enhancement, even the storage period increased. Furthermore, for detection and removal of certain harmful substances that deteriorate the quality and shelf life of milk and cheese, nanomaterials such as metal nanoparticles, quantum dots, engineered nanoparticles, and nanotubes were used. Lastly, for the packaging mostly nanoparticles and nanocomposites were used, along with protection against environmental factors; these nanomaterials improved the quality of dairy products. Altogether, it was noticed that nanomaterials can have more than one benefit of the application in the dairy sectors. Nanomaterials serve the purpose of revolutionizing several aspects associated with dairy industries. In conclusion, nanotechnology has the potential to become the most feasible, safer, better, and consumer-accepted technology in the immediate future.

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Chapter 26

Development of biosensor-based technology for the detection of pathogenic microorganisms and biomolecules in dairy products

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26.1 Dairy products and microorganisms

Milk and dairy products, being rich in lipids, proteins and sugars, are the excellent growth media for the microorganisms. Beneficial microorganisms such as lactobacilli and bifidobacteria, present in the milk and dairy products, help in digestion, protect against infections caused by the pathogenic microorganisms, and help with lifestyle disorders such as obesity, diabetes, non-alcoholic fatty liver diseases, etc. (Amara & Shibl, 2015; Koutnikova et al., 2019) Lactobacilli and bifidobacteria are probiotic microorganisms normally found in the human gastrointestinal tract. Along with beneficial microorganisms, some pathogenic microorganisms such as *Staphylococcus aureus, Salmonella* spp., *Listeria monocytogenes, Escherichia coli* O157:H7 and *Campylobacter* are also present in milk and dairy products, and are the cause of the foodborne diseases linked to raw milk (Claeys et al., 2013; Kousta et al., 2010; Yang et al., 2012) and raw cheese (Verraes et al., 2015) in humans. Contaminated sources in dairy farms and diseased farm animals are the potential sources of the pathogenic microorganisms in dairy products. Types of the microorganisms found in the milk are affected by many factors such as microorganisms found in the teat canal, on the surface of teat skin, in the surrounding air, feed of farm animals, water supply quality, and hygienicity of the milkmaids and the dairy equipment (Quigley et al., 2013). Although pathogenic microorganisms present in the milk are killed either through high-temperature short-time (71°C−72°C for 15 seconds) pasteurization or low-temperature long-time pasteurization (61.5°C for 30 minutes), however, certain thermoduric microorganisms such as some species of genus *Bacillus, Micrococcus, Corynebacterium* and *Enterococcus* can survive the pasteurization process, and are transmitted to pasteurized milk (Thomas & Prasad, 2014). In developing countries like India, due to improper handling, processing, and storage of dairy products, certain pathogenic microorganisms grow postpasteurization; consequently, chances of transmission of pathogenic microorganisms are always at high risk. This could be responsible for many diseases like brucellosis, listeriosis, tuberculosis, etc. (Dhanashekar et al., 2012). Therefore, it is of utmost importance for the dairy industries to ensure the elimination of these pathogenic microorganisms from the dairy products before being dispatched to market for the human consumption.

Conventional methods such as plate count method, chromatography, enzyme-linked immunosorbent assay (ELISA), spectroscopy, electrophoresis, polymerase chain reaction (PCR), titrations, etc., are available for the microbial analysis of food products, but these methods are slow, expensive; in some cases, sample extraction and purification is required by a trained operator, and real time monitoring is not possible (Meshram et al., 2018). Moreover, some components such as fat, calcium, and indigenous enzymes found in dairy products are the potential inhibitors that have a negative effect on the accuracy and sensitivity of the test used for the detection of pathogenic microorganisms (Cancino-Padilla

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et al., 2017). So, it is the need of today to develop fast, reliable, sensitive, specific, automated and cost-effective methods to detect foodborne pathogens in dairy-based products.

26.2 Traditional methods for detection of pathogenic microorganisms in dairy products

Different microbiological methods are available for the detection of the pathogenic microorganisms in the dairy products, and each method is associated with its own advantages and disadvantages.

26.2.1 Culture-based conventional methods

Culture-based conventional methods are considered to be gold standard methods for the detection of microorganisms as they are easy to perform, sensitive, cost-effective and have the ability to confirm cell viability (Senturk et al., 2018). Anderson et al. (2011) examined the microbial quality of pasteurized milk purchased from supermarkets in a developing country (Jamaica) using tests such as methylene blue reduction test, standard plate count, coliform plate count, purity plate culture, Gram's staining and biochemical tests. Most of the samples tested contained undesirable levels of *Enterobacter* spp. and *Escherichia coli*. Culture-based conventional methods have some disadvantages like (1) time consuming, (2) skilled staff is required, (3) chances of contamination during different steps of the process are more, and (4) different media is required for different types of microorganism.

26.2.2 Polymerase chain reaction

PCR is the most reliable, accurate, sensitive, specific and versatile nucleic-acid amplification based method for the detection, identification and characterization of pathogenic microorganism in the dairy products. Allmann et al. (1995) analyzed the raw milk samples and dairy products made from raw milk for the detection of pathogenic microorganisms using PCR. *Listeria monocytogenes* was detected and identified by amplifying the DNA sequence of *hly* gene; *E. coli* was identified by amplifying *malB* gene; *E. coli* LTI (heat-labile toxin type I) enterotoxigenic and *E. coli* STI (heat-stable toxin type I) enterotoxigenic were identified by amplifying genes *elt* and *est*, respectively; and *Campylobacter jejuni* and *Campylobacter coli* were identified by targeting flagellin genes *A* and *B* (*fla A*/*fla B*). Non-availability of primer pairs for every pathogenic microorganism is the major disadvantage of the PCR method. Sometimes, "false positive" and "false negative" results are obtained. Therefore, further confirmation of the results is required (Senturk et al., 2018).

26.2.3 Enzyme-linked immunosorbent assay

Pathogenic microorganisms cause illnesses through the production of toxins in food products. These toxins can be determined qualitatively and quantitatively, through the technique called ELISA. This technique is reliable, precise and results can be obtained in 3−4 hours. But sensitivity of this technique is low (Senturk et al., 2018). Shen et al. (2014) detected the *E. coli* 0157:H7 in artificially contaminated milk, vegetable and ground beef with ELISA in 3 hours. The detection limit of ELISA was reported to be 68 CFU/mL in PBS and 6.8×10^3 CFU/mL in food samples.

Therefore, it is of utmost important to develop simple, cheap, selective, sensitive and reliable analytical methods for the detection of the pathogenic microorganisms to avoid transmission of milk borne pathogens to humans, and biosensors are being considered an effective tool in this microbial analysis of food products.

26.3 Biosensors

A typical biosensor is made up of two components viz. bioreceptor (biological element) and transducer. Bioreceptor is the part which interacts with the analyte through specific biological interactions. Biological molecules viz. antibodies, proteins, enzymes, nucleic acids, cells, wholes tissues and microorganisms can be used as bioreceptor. Transducer is the part which converts the biological interactions into the measurable signals which are read by observer or instrument. Transducers are of different types viz. optical, electrochemical, piezoelectric, thermometric etc. Electrochemical and thermometric transducers are used to detect enzymatic reactions; whereas, mass sensitive reactions are detected by piezoelectric transducers (Meshram et al., 2018). Several kinds of biosensors can be made using different combinations of bioelements and transducers to suit different applications.

Biosensor is an analytical device which is used to detect unwanted microbial and chemical agents in environmental pollution, food industry, pharmacy, biotechnology, agriculture, mining, military defense and country security (Liu & Lin, 2005; Sadana, 2006). Because of their high selectivity, biosensors can be used for the direct analysis of complex samples such as milk with or no minimal sample purification. Despite of their diverse use in the analysis, use of biosensors is limited to research laboratories only. X-MARK™ (nanoRETE Inc.) and Aegis1000 (Bio Detection Instruments) are commercially available biosensors for the detection of foodborne pathogens (Mortari & Lorenzelli, 2014).

26.3.1 Ideal biosensor

An ideal biosensor should have following features/characteristics:

1. It should be highly selective in detecting the target analyte.
2. It should not show any cross-reactivity with other molecules having structure similar to target analyte.
3. Activity of biological element should be retained for long time.
4. Sensitivity of biosensor should be high.
5. Minimum or no processing of the samples should be required during execution.
6. It should be stable in various environmental conditions during analysis.
7. Results or signals produced by biosensor should be reproducible.
8. Results should be quick so that real time monitoring of the target analyte can be done.
9. It should recover itself very soon after execution so that it can be reused.
10. Cost of the instrument and cost per test should be less.
11. It should be fully automated and minimum operator skill should be required.
12. Response of the biosensor should be linear and it should cover the concentration range of the analyte.

26.3.2 Methods of immobilization of bioelement onto transducer

Immobilization of bioelement onto transducer is very crucial step for biosensor to work efficiently. Immobilization process should not affect the biological activity of the biomolecule (bioelement). Immobilized biomolecule should be fully accessible to analyte to be measured, and remain stable for long term. Transducer should not be affected during immobilization process. Many immobilization methods viz. membrane entrapment, physical adsorption, matrix entrapment, covalent binding, electrochemical polymerization and photo-polymerization are available, each has its own advantages and disadvantages. Therefore, immobilization method to be adapted depends upon transducer, bioelement and other assay requirements (Kuhnert et al., 2000).

26.3.2.1 Physical adsorption

In this method, biomolecule is adsorbed onto solid surfaces such as cellulose, collagen, PVC, gold and carbon via weak interactions such as ionic interactions, hydrogen bonds, van der Waal's forces and hydrophobic interactions. In this method, biomolecule to be adsorbed is incubated with solid surfaces for certain time period. Thereafter, excess biomolecules are removed by washing. However, stability of adsorbed biomolecule layer onto solid surface is poor, and can be affected by pH, temperature and ionic strength. Stability of adsorbed biomolecule can be increased by cross-linking using bifunctional reagents e.g., glutaraldehyde. But cross-linking of biomolecules can sometimes affects the activity of biomolecule (Canhoto & Magan, 2003).

26.3.2.2 Encapsulation or confining

This method is mainly done for enzymes. In this method, enzymes are confined in microcapsules made up of either semipermeable membrane or liposomes. Substrates and products can cross the semipermeable membrane such as dialysis membrane but enzymes can't leak out of this membrane. Immobilized enzymes in the microcapsules are difficult to get associated with transducer (Mello & Kubota, 2007).

26.3.2.3 Covalent binding

Biomolecule can be immobilized onto solid surfaces by covalent linkages. Proteins/enzymes as biomolecules are attached on the activated solid surfaces via their amino acid residues groups like thio, carboxyl, phenolic, imidazole,

disulfide, hydroxyl and thioether groups. The prerequisite for covalent binding is the presence of functional groups on the solid surfaces. These functional groups are either be the integral part of the solid surfaces or can be introduced on the solid surfaces by using another activated matrix or membrane. Many different preactivated membranes are available commercially. Type of the membrane to be chosen is based on type of functional group present on the surface of protein to be immobilized (Mello & Kubota, 2002).

26.3.2.4 Entrapment

Enzymes along with mediators and additives are physically entrapped in three dimensional networked polymeric gels viz. starch gels, polyacrylamide gels, silica gels, etc. During this immobilization method, modification of bioelement does not takes place, therefore, activity of the bioelement is not affected at all. Moreover, biosensors based on this immobilization method have shown enhanced operational and storage stability. However, leaching of the bioelement can hamper the performance of the biosensors (Sassolas et al., 2012).

26.3.2.5 Electrochemical polymerization

Firstly, transducer is soaked in an aqueous solution having enzymes and monomeric molecules, thereafter, an appropriate electric current or potential is applied to the transducer. Due to electric current or potential monomeric units get oxidized that results into formation of radical cation. These radical cations react with another radical cation or neutral monomer to form an oxidized dimer. Oxidized dimer reacts with another neutral monomer or radical cations and so on. This ultimately forms a polymer at the electrode surface. Enzymes near the surface of electrodes are physically incorporated into growing polymer network (Sassolas et al., 2012).

26.3.3 Generations of biosensors

26.3.3.1 First generation biosensors

Bioelement and transducer components of the biosensors can be easily separated from each other. Removal of one component does not affect the functioning of other component. In these biosensors, electrons produced during the electrochemical reactions are transferred to electrode using molecular oxygen. Therefore, these biosensors are also called as mediator less biosensors. Enzymes (oxidases and dehydrogenases) immobilized onto the transducer convert the substrate into electroactive measurable product. These biosensors estimate the conc. of analyte by measuring the decrease in conc. of oxygen and/or increase in conc. of hydrogen peroxide.

26.3.3.2 Second generation biosensors

Bioelement and transducer components of these biosensor are attached to each other in a more intimate fashion. Removal of one component can affect the functioning of other component. In these biosensors, electrons produced due to electrochemical reaction are transferred to electrode using artificial mediators or nanomaterials. Therefore, these biosensors are also called mediator biosensors. Ferricyanide and ferrocene are the example of common and well-known mediators. Other mediators such as methylene blue, methyl violet, thionin, toluidine blue, alizarin yellow, phenazines, prussian blue and inorganic redox ions can also be used. Mediator can either be added to the sample or be immobilized near to the enzyme on the electrode (Chaubey & Malhotra, 2002).

26.3.3.3 Third generation biosensors

Third generation biosensors depend upon bioelectrocatalysis (Dzyadevych et al., 2008) where electrons produced by electrochemical reactions are transferred directly to the electrode without any mediators or molecular oxygen. These biosensors are made of three elements viz. enzyme as bioelement, redox polymer or nanoscale wiring element and the electrode. The function of redox polymers or nanoscale wiring element is to transfer the electrons from the redox center of the enzyme to the electrode. Third generation biosensors are not used extensively for sample analysis as the development of these biosensors is still the topic of ongoing research (Rocchitta et al., 2016). Term biochip is used to describe such kind of instrument.

26.3.4 Types of biosensors

26.3.4.1 Electrochemical biosensors

Electrochemical biosensors are inexpensive and best suited for the analysis of turbid samples such as milk. Therefore, electrochemical biosensors are used commonly for milk analysis, followed by optical and piezoelectric biosensors (Mortari & Lorenzelli, 2014). Electrochemical biosensors can be categorized as (1) potentiometric biosensors, (2) amperometric biosensors, (3) conductometric biosensors, and (4) impedimetric biosensors. Among them amperometric and potentiometric biosensors are most common.

26.3.4.2 Amperometric biosensors

Amperometric biosensors measures the electric current at constant potential. Electric current generated due to the production and consumption of electrochemical species by the bioelement part of the biosensor. Signal displayed by the biosensor is proportional to the conc. of the analyte. Two or three electrodes are used in these biosensors. Lin et al. (2008) developed a disposable amperometric immunosensing strip for the detection of *E. coli* O157:H7 via an indirect sandwich enzyme-linked immunoassay. Briefly, primary antibodies specific to *E. coli* O157:H7 along with mediator (ferrocene dicarboxylic acid) were immobilized on the working electrode. Gold nanoparticles (13 nm diameter) were immobilized on working electrode for signal amplification. After capturing of bacteria by immobilized primary antibody, horseradish peroxidase labeled secondary antibodies were added. Oxidation of substrate such as hydrogen peroxide by the enzyme generated the electrical current proportional to the bacterial conc. *E. coli* O157:H7 in the range of 10^2-10^7 CFU/mL could be detected using these immunosensing strips. The detection limit of these strips was reported to be approximately 6 CFU/strip in PBS and 50 CFU/strip in milk. Alexandre et al. (2018) developed the amperometric biosensor for the detection of *S. typhimurium* in milk. The detection limit was reported to be 10 CFU/mL.

Detection of lactose concentration in raw milk by amperometric biosensor

Lactase enzyme, produced from the human intestinal epithelium cells, breaks down the lactose into glucose and galactose. In lactose-intolerant humans, activity of lactase enzyme decreases drastically. Consequently, unmetabolized lactose reaches to large intestine. Gut microbiota splits the lactose into organic acids viz. acetic acid, butyric acid, propionic acids and other organic acids. These organic acids are absorbed and used by intestinal cells. Some gases viz. methane, hydrogen and carbon dioxide are also produced due to microbial activity on the lactose. These gases cause the bloated, gassy and nauseous feeling (Friedl, 1981; Suarez et al., 1995). Therefore, lactose-intolerant humans are discouraged to consume lactose containing products, majorly milk. Therefore, it is very important to detect lactose in the milk and other dairy products before being consumed by the lactose-intolerant humans. Biosensors seems to be very effective tool for determining lactose in the milk as available traditional methods viz. spectrophotometry, chromatography, polarimetry etc. are very tedious and time-consuming (Sharma et al., 2007).

Eshkenazi et al. (2000) developed amperometric biosensor for the determination of lactose conc. in the diluted fresh raw milk added with 5-aminosalicylic acid. In this kind of biosensor, three enzymes viz. beta-galactosidase (converts lactose into glucose and galactose), glucose oxidase (converts glucose and molecular oxygen into gluconic acid and hydrogen peroxide) and horseradish peroxidase (reduces the hydrogen peroxide into water) are immobilized over the glassy carbon electrode. Three cascaded enzymatic reactions enhance the sensitivity and selectivity of the biosensor. Sharma et al. (2007) developed the lactose biosensor by immobilizing lactase (beta-galactosidase) and galactose oxidase (oxidation of D-galactose into D-galacto-hexodialdose and reduction of oxygen into hydrogen peroxide) enzymes on polyvinyl formal membrane. These enzymes immobilized membrane was attached to the electrode of dissolved oxygen analyzer. However, this kind of biosensors are not recommended for solutions containing galactose, especially hydrolyzed milk as gives false positive results.

26.3.4.3 Potentiometric biosensors

Potentiometric biosensors measure the oxidation-reduction potential of an electrochemical reaction. Ion sensitive electrodes are used in potentiometric biosensors to detect the changes in the concentration of specific ion during a biochemical reaction. Antigen-antibody reaction induces the small change in the charge of the protein which can be detected using potentiometric biosensors. Potentiometric biosensors are not sensitive as change in charge of the protein is too small (Senturk et al., 2018). Zelada-Guillén et al. (2010) developed the potentiometric biosensor for the detection of *E. coli* in semi-skimmed milk in a few minutes. Aptamers (oligonucleotide or peptide molecules that bind specifically to target analyte) chemically linked to carbon nanotubes were used as biorecognition element for the detection of specific strain of *E. coli* in complex samples. Biosensor can detect the living bacteria at conc. levels as low as 6 CFU/mL for complex matrices such as milk.

Guilbault and Montavelo in 1969 developed first potentiometric enzyme-based biosensor for the measurement of glucose levels in the sample using enzyme glucose oxidase immobilized on the electrode. Later, other enzyme-based biosensors have been developed using various enzyme such as urease, lactate dehydrogenase and glutamate dehydrogenase (Senturk et al., 2018). In enzyme-based potentiometric biosensors, the change in the pH during a biochemical reaction is detected by pH sensitive electrodes.

Detection of urea in the milk by potentiometric biosensor

Urea forms the major non-protein nitrogen in the milk. Accepted urea conc. in the milk is 70 mg/dl. Urea conc. above this level can cause several problems viz. indigestion, acidity, ulcers, cancer and renal problem in humans. For commercial benefits urea, caustic soda, refined oil and detergents are used to prepare synthetic milk. Therefore, dairy industries are developing methods for the detection of urea in the milk, and biosensors seems to be an effective tool.

Urea present in the sample is broken down into CO_2 and NH_3 by the urease enzyme. At pH 7.0, in aqueous medium, CO_2 and NH_3 dissociates as:

$$CO_2 + H_2O \text{----------} HCO_3^- + H^+$$

$$NH_3 + H_2O \text{----------} NH_4^+ + OH^-$$

Using a variety of transducers viz. pCO_2 electrode, pNH_3 electrode, pH electrode and pNH_4^+ electrode, urea conc. can be determined potentiometrically (Trivedi et al., 2009).

Urease enzyme is entrapped in polymeric gel, and immobilized over ammonium sensitive electrode. This enzyme immobilized ammonium sensitive electrode is used for the detection of urea conc. in the sample (Trivedi et al., 2009). However, this kind of biosensors are highly sensitive to monovalent cations such as $H+$, $K+$ and $Na+$ ions, therefore, buffer solution whose constituents don't affect the enzyme electrode is required (Trivedi et al., 2009).

26.3.4.4 Optical-based biosensors

In this type of biosensors, biorecognition element is attached to optical transducer system. Optical biosensors emit the optical signals proportional to the concentration of analyte in the sample. First commercial available optical biosensor was fiber optics (Senturk et al., 2018). Sensitivity of optical biosensors are higher than other biosensor but these biosensors can't be used for turbid samples such as milk. BioFlash is the commercial available optical biosensor developed by Innovative Biosensor Environment Group Inc. (Mortari & Lorenzelli, 2014).

BIOCORE Q (BioCore Inc.) and Spectra™ (Sensata Technologies Inc.) are commercial available Surface Plasmon Resonance (SPR) biosensors and are used for the detection of foodborne pathogens (Chinowsky et al., 2003). Raghu and Kumar (2020) developed a novel SPR biosensor for the detection of *L. monocytogenes* in milk samples using wheat germ agglutinin (WGA) as a bioelement. Selective interaction of WGA lectin with surface carbohydrate component of *L. monocytogenes* was the used as biorecognition event. Eser et al. (2015) detected the *Salmonella enteritidis* with high specificity in milk sample using SPR biosensor. Antibody against the *S. enteritidis* was used as bioelement. Detection limit of this biosensor was reported to be 1×10^2 CFU/mL.

26.3.4.5 Mass sensitive biosensors or piezoelectric biosensors

Piezoelectric quartz crystal microbalance (Chen et al., 2008; Shen et al., 2011) and magnetoelastic biosensors (Guntupalli et al., 2007; Lakshmanan et al., 2007) have been used for microbial analysis of milk samples. Shen et al. (2011) used the piezoelectric quartz crystal microbalance for the detection of *E. coli* 0157:H7 in milk using antibodies as a biorecognition element. The detection limit of biosensor was found to be 53 CFU/mL, and total assay time was 4 hours. In another study, *Salmonella typhimurium* was detected in the fat free milk using bacteriophage as biorecognition element by magnetoelastic biosensor. The detection limit of this biosensor was 5×10^3 CFU/mL, and total detection time was 20 min for analysis of 1 mL sample (Guntupalli et al., 2007; Lakshmanan et al., 2007). Lian et al. (2015) used the piezoelectric biosensors for the detection of *S. aureus* in culture and milk, and detection limit was ranged in between 4.1×10^1 to 4.1×10^5 CFU/mL. In another study, *L. monocytogenes* were detected in the milk sample by piezoelectric biosensor with detection limit of 10^2 CFU/mL (Sharma & Mutharasan, 2013).

26.3.4.6 Thermometric biosensors

These biosensors measure the heat (enthalpy) change during the biochemical reaction. Heat change is accompanied by a change in the temperature of the reaction medium. As temperature change is very small, sensitive thermistors are used to monitor the temperature change during the biochemical reaction. Sometimes, heat change of reaction is

amplified by coupling the reaction with another reaction accompanied by a higher heat change (Meshram et al., 2018). Extensive use of thermometric biosensors for the detection of milk borne pathogens have not been reported till now.

In conclusion, foodborne diseases are rising at an alarming rate both in developed and developing countries. Different advanced methods for the detection of pathogens in dairy products have been developed. Biosensors are fast, reliable, and economical analytical tools for the accurate detection of pathogens and biomolecules in dairy products. Different types of pathogenic microorganisms (bacteria, fungus, viruses) and biomolecules (urea, lactose, glucose) are found in the dairy products; therefore, different types of biosensors are required to detect them. Further research is required to develop multifunctional and versatile biosensing systems for the detection of multiple microorganisms and biomolecules using a single device in the sample.

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Chapter 27

Machine Learning applications in dairy farm management

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27.1 Introduction to dairy farm management

Dairy farm management incorporates a wide scope of exercises. Choices concerning these exercises are made consistently, regularly utilizing loose strategies or inadequate data, which can result in imperfect outcomes. Computer-based instruments can be utilized to help the choice cycle, in this manner improving the effect and after-effects of the choice. Apportion adjusting and sire determination are two areas in which computer-based apparatuses have increased greatly in use far and wide. A general new strategy in choosing emotionally supportive networks is the utilization of information bases. Utilization of these rising methodologies can profit the client and the engineer by giving stronger applications that are less expensive to keep up (Adesh et al., 2005).

The technique by which knowledge is spoken to in a knowledge base, that is, the "knowledge portrayal conspire", can change. A wide range of knowledge portrayal plans have been or are being created for explicit spaces. Each knowledge portrayal technique has its own qualities and shortcomings for the attributes of the knowledge to be demonstrated. The knowledge portrayal conspires chosen for an emotionally supportive network has ramifications for the exhibition of the framework and the execution of its assignment. Knowledge associated with one part of dairy farm management frequently has attributes not quite the same as knowledge in different parts. Subsequently, knowledge for a particular emotionally supportive network for dairy farms may require more than one portrayal technique (Adesh et al., 2005).

Different creators have portrayed the utilization of knowledge-based frameworks (KBS) in farming management. Albeit different knowledge portrayal plans exist, most depictions are restricted to run-based frameworks. No outline is accessible of the qualities of knowledge portrayal plans corresponding to their conceivable use in dairy farm management support. In this manner the goals are to depict different knowledge portrayal techniques with their qualities and shortcomings, and to give instances of their application to dairy farm management. Accentuation is on techniques for which item advancement apparatuses are accessible.

27.2 The state of art of dairying in developing countries

From the recorded perspective, the beginning of dairying lies in the agricultural nations, in Mesopotamia to be exact, at around 6000−7000 BCE. From this area, milk creation and milk utilization spread to different locales in Europe, North and East Africa, and Asia. The non-industrial nations can be partitioned into customary and non-conventional milk makers. Conventional milk-creating areas are, generally, the nations of the Mediterranean and the Middle East, the Indian subcontinent, the Savannah districts of Western Africa and the Highlands of Eastern Africa, and somewhat South and Central America. Further, the utilization of milk and dairy items assumed a significant job among the migrants in Africa and Asia. The regional population of South East Asia, China, Korea, and Japan, represent the non-customary milk-creating nations. All things considered, for instance in China, milk was viewed as gainful for the evil and the older (Provost & Fawcett, 2007).

In the "conventional" milk-creating districts in Asia and mostly in Africa, the structure of milk creation is described by little homesteads with not more than three or four creatures. Dairying there is almost in every case part of a blended

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cultivating framework. The dairy steers are regularly utilized as draft creatures also. Domesticated animals are taken care of primarily on rural buildups and squander, and are groomed on common fields of non-arable land (Tiezzi et al., 2001). Cows husbandry and milk creation are generally upheld on the results of agribusiness. Thusly, a healthy prevalent item is created in a biologically and naturally good way.

In Central and South America, the scale and plan of dairying are blended with agricultural and dairy tasks. The normal milk creation per cow is higher than in the areas referenced above, at around 1000 kg for every year, except it goes from 1400 to 1900 kg in Chile and the Eastern piece of the Argentine, the entire of Uruguay, and the southern piece of Brazil (Caraviello et al., 2001). All things considered, here, too, the small milk maker likewise assumes a significant job. Evaluations show that in most of the Latin American and Caribbean nations between 60% and 80% of the milk makers can be considered limited scope makers, representing 25%−30% of milk creation in these nations.

In the "non-conventional" milk-creating nations the structure of dairying is different. Particularly in the tropical and subtropical areas, other than little homesteads, there are huge, specific dairy ranches, at times with a few hundred bovines or more; the majority of which were established in frontier times or after the Second World War (Berry et al., 2003). In some economies, there are regularly still huge capital-serious and specific state ranches, for instance in Cuba, China, Ethiopia, and Tanzania (Cestnik, 1990). Saudi Arabia, for example, has a huge scope of dairy ranches with up to a few thousand dairy bovines.

27.3 Knowledge characteristics for dairy management

Knowledge incorporates realities about the issue and a wide cluster of critical thinking techniques that a specialist collects after some time. To tackle an issue, two classes of knowledge are frequently important: (1) knowledge about realities in the area, declarative knowledge, and (2) knowledge of how to utilize this declarative knowledge: procedural or operational. The two kinds of knowledge have their own highlights and are depicted next.

27.3.1 Declarative knowledge

At the farm level, declarative knowledge incorporates perceptions made on a farm or known to be significant for the farm venture. These perceptions can be made by people or via programmed gadgets (sensors) (Cornwell & Nebel, 1989). Data gathered naturally are sometimes preprocessed before they are put away and prepared for use. Declarative knowledge can be accessed either from an on-farm or off-farm database (DeJarnette, 2000). Two significant highlights of declarative knowledge can be portrayed as far as culmination and sureness. At the point when choices are being made on a dairy farm, knowledge is frequently deficient, questionable, or both.

27.3.1.1 Culmination

Inadequacy alludes to the extent of perceptions that are absent. For instance, when perceptions are made utilizing mechanized sensors (e.g., electrical conductivity estimations of the milk), failing gear can prompt missing perceptions.

27.3.1.2 Assurance

Perceptions can be viewed as certain. In any case, during dynamic, perceptions frequently must be converted into more broad terms that are utilized as a base to draw conclusions (Gianola & Rosa, 2004). For instance, it is helpful to know whether a creature has a fever, an internal heat level >39°C. A contrast of just 0.2°C (38.9°C vs 39.1°C) can recognize a derivation of fever or no fever. An observer would be surer of a fever from a sensor indicating 43°C than from an indication of 39.1°C. Hence, the portrayal of knowledge utilizing soft limits is frequently better (González-Recio, 2005; Grzesiak & Lacroix, 2005), for example, 39.1°C would be classed as a fever with less sureness than 43°C.

27.3.2 Procedural knowledge

Procedural knowledge utilizes perceptions and data and changes them into data that is valuable to the client. The procedural knowledge in regular PC programs, for example, a management data framework, revamps and joins the different perceptions to make them simpler to decipher (Sheldon et al., 2001). The procedural knowledge in KBS tries to help take care of explicit issues. The procedural realization has three qualities: (1) generality, (2) certainty, and (3) knowledge level.

27.3.2.1 Generality

Procedural knowledge can be general or explicit. Explicit knowledge can comprise, for instance, the relationship between a difficult circumstance and an answer. These affiliations are frequently evolved for a fact and some of the time portrayed as dependable guidelines. A case of associative knowledge is the utilization of anti-infection treatment (Kononenko, 1990). An accomplished veterinarian realizes what anti-infection to recommend in which circumstance without speculation about the specific component of activity of that anti-toxin. When such heuristic knowledge is spoken to, it tends to be used consistently without understanding the basic instruments of activity.

Rather than heuristic knowledge, conventional knowledge comprises a causal clarification for the unfortunate attributes of that circumstance. The cause of everything leads to the quality of that thing. Here, cause and quality is linked. The knowledge used to produce a causal clarification of an issue is broader; the knowledge can likewise be utilized to clarify the functions of a framework or to reenact the conduct of a framework.

27.3.2.2 Certainty

Similarly, as with declarative knowledge, certainty is also a significant component in procedural knowledge. Thinking under uncertainty is basic in finding an illness. Sickness analyzed by specialists regularly takes a subjective structure, counting uncertainty (e.g., *Staphylococcus aureus* is the most probable microbe causing this particular mastitis case). The choice cycles hidden by these conclusions comprise procedural knowledge with differing degrees of uncertainty. Uncertainty is normal in natural frameworks where exact knowledge concerning instruments of activity for dairy animals are restricted. Complete certainty can be thought of as an exceptional sort of uncertainty (Kush, 2005).

27.3.2.3 Knowledge level

A computer-based framework playing out a particular undertaking has different utilitarian levels. The most reduced level is the gadget level or spot level (Liu & Reents, 1995). The most significant level in a conventional computer-based program is the program, or emblematic level, and is justifiable by the vast majority with programming experience. Images are portrayals of certifiable items. A Knowledge-Based System (KBS) presents another computational level over the image level: the knowledge level (Long & Avendaño, 2008). A knowledge portrayal plan can be viewed as a decrease of knowledge from the knowledge level to the lower image level.

Most knowledge can, without much of a stretch, be spoken to in images; that is, it can undoubtedly be visualized, starting with one individual then onto the next. Knowledge that can undoubtedly be moved into images is characterized as representative knowledge.

Sub-symbolic knowledge is knowledge that can't be visualized effectively in images; that is, sub-symbolic knowledge is hard to disclose to other people. The knowledge associated with design acknowledgment is viewed as sub-symbolic knowledge. For instance, in the investigation of milking bends, the dairy farm guide can perceive issues in milk creation right away. In any event, when the data in the bend are not finished, clarifying why a specific bend demonstrates a milk creation issue takes significantly more time. Knowledge engaged with bend understanding along these lines can be considered as sub-symbolic knowledge.

27.4 Methods of knowledge representation for dairy management

Different knowledge portrayal strategies have been created for use in choosing emotionally supportive networks. A few strategies included and embraced for the public area, shareware, or business programming bundles can be utilized to encourage the advancement of choice emotionally supportive networks. Different techniques are presently being worked on in artificial intelligence research labs, and those strategies are regularly fit uniquely for one topic space (Lowed & Domingos, 2005). The knowledge portrayal techniques that have item advancement programming and that give off an impression of being promising for use with dairy science applications are depicted in the accompanying segments. These chosen strategies and their different qualities and shortcomings for declarative and procedural knowledge attributes are summed up as shown in Fig. 27.1.

27.4.1 Production rules

The most popular and most applied knowledge portrayal conspired is the utilization of creation frameworks. Procedural knowledge in a creation framework is spoken to by a lot of rules by which the area procedural knowledge is consolidated: a database of space declarative knowledge and a derivation component for applying the rules to the database

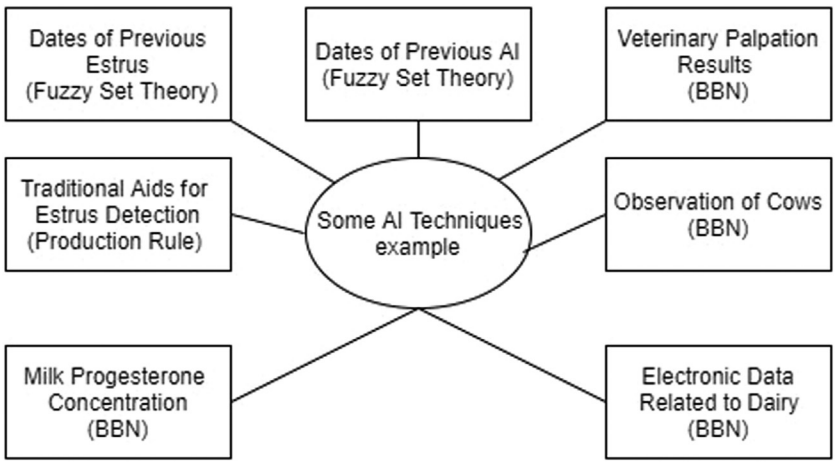

FIGURE 27.1 Artificial intelligence techniques used in dairy farm management.

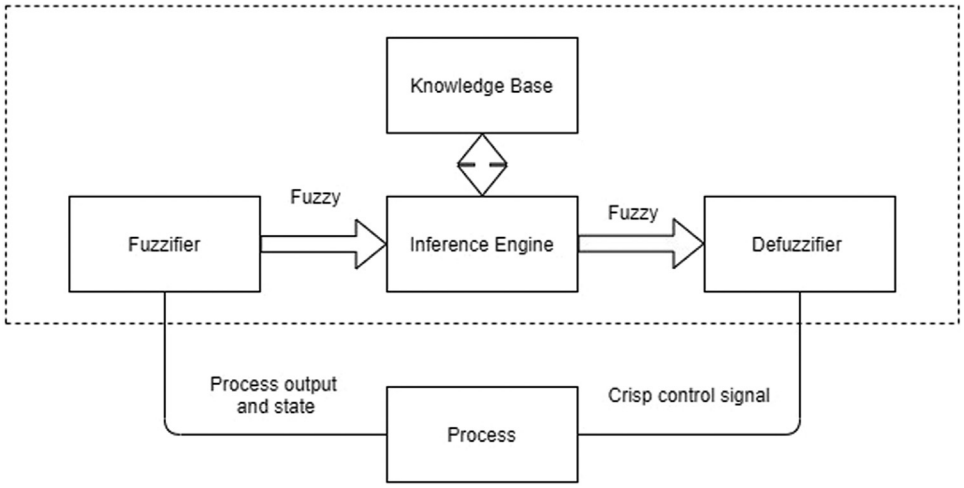

FIGURE 27.2 Fuzzy logic implementation.

(Justyna et al., 2016). The standards (ifat that point rules) speak to condition and activity sets. The forerunner (if) of a standard is a condition for the standard to be relevant, and the ensuing (at that point) of a standard is the activity that results when the standard is applied (Oikonomou & Banos, 2007). Forward and reverse thinking are the most well-known derivation systems. Creation rules are extremely proficient in speaking to heuristic knowledge, however complete declarative knowledge is expected to take care of issues. A few improvement instruments, called shells, in light of creation frameworks, are financially accessible. Since an assortment of shells is accessible, Lowed depicted in his paper the least guidelines for the user interface (UI) for creating framework advancement devices (Lowed & Domingos, 2005). Creation frameworks have been utilized in dairy choice emotionally supportive networks to dissect yearly financial executions, assess regenerative execution, milk creation execution, and correlations between wanted (arranged) results with real outcomes.

27.4.2 Fuzzy logic

Using the fuzzy set hypothesis, factors can be related with a collaborative work that can take values somewhere in the range of 0 and 1 to depict the significance of the variable. The essential highlights of the fuzzy set hypothesis can be characterized as follows. In the event that S is a set, and s is an individual from that set, a fuzzy subset 0 is then characterized as a membership work mF(s) that describes how much s has a place with F. Utilizing predefined membership capacities, it tends to express how fever is detected at the p point at which a temperature of 39.1°C is watched (Shahinfar & Weigel, 2010). At the point when the fuzzy set hypothesis is applied in a numerical or PC framework, it tends to be alluded to as fuzzy logic shown in Fig. 27.2.

Fuzzy logic is regularly utilized with creation rules to join proportions of probability. These highlights make fuzzy logic helpful in circumstances which details characterizing qualities of significance, however, not straightforwardly noticeable highlights is troublesome. Various choice instrument improvement frameworks that use fuzzy logic are monetarily accessible. Albeit fuzzy logic is basically applied in regulator errands, it is being applied all the more often in choice emotionally supportive networks. In Japan, the fuzzy set hypothesis is applied to dairy farm financial aspects.

27.4.3 Bayesian belief network

The hypothesis of Bayesian belief networks (BBN), otherwise called "causal probabilistic networks", depends on Bayesian conditionalization. A BBN is, subjectively, a chart on which the hubs speak to space objects and the connections between hubs speak to relations between these items (Uusitalo, 1965). The knowledge is expressed in a causal way: for instance, sicknesses cause indications. Every hub in a BBN has various states, depicting the potential estimations of the hub. Quantitatively, the connections communicated by the connections are spoken to by conditional probabilities as shown in Fig. 27.3.

In a BBN, the conditional probabilities for every hub on its folks [for this situation P(BIA)] are put in a probability table. At the point when the condition of hub B has been watched, the conditional probability P(AIB) can be determined utilizing the probability table. A BBN can be made with the decision to uphold the advancement instrument, HUGIN (Hugin Expert A/S, Aalborg, Denmark) (Uusitalo, 1965). A BBN, which is exceptionally valuable in displaying uncertainty, can likewise dissuade fragmented knowledge. In dairy science, BBN has been utilized to inspect the cow's breeding, to analyze mastitis brought about by natural elements, and to decide the blood gathering of Danish Jersey cows.

27.4.4 Conditional causal model

A conditional causal model (CCM) comprises a lot of hubs that portray an area. The hubs are associated by a lot of unidirectional connections, speaking to a causal reliance of a hub on another hub. The greatness of the reliance might be impacted by at least one condition. The connections in a CCM might be subjective and quantitative. Hub B is causally subject to hub A, a relationship spoken to by the unidirectional bolt. Hub C is a condition, spoken to by a hover on a bolt. Forward reasoning (simulation) and backward reasoning (diagnosis) are conceivable with a CCM. A CCM is truly adaptable and permits the conventional portrayal of complex procedural knowledge. Data or perceptions utilized for input should be finished, and a CCM permits no reasoning with uncertainty as shown in Fig. 27.4.

A CCM can be created utilizing the apparatus CAMEL (causal modeling environment and laboratory; Laboratory for Artificial Intelligence, Erasmus University, Rotterdam, The Netherlands) (de Vries & Veerkamp, 2000). Utilizing the graphical interface, models in a space can be made. The connections between the hubs would then be able to be evaluated with fundamental capacities, written in the artificial intelligence programming language Common LISP. CCM is being utilized for the diagnosis of crowd mastitis issues. CCM is utilized to help decisions with respect to take care of grassland use on dairy farms.

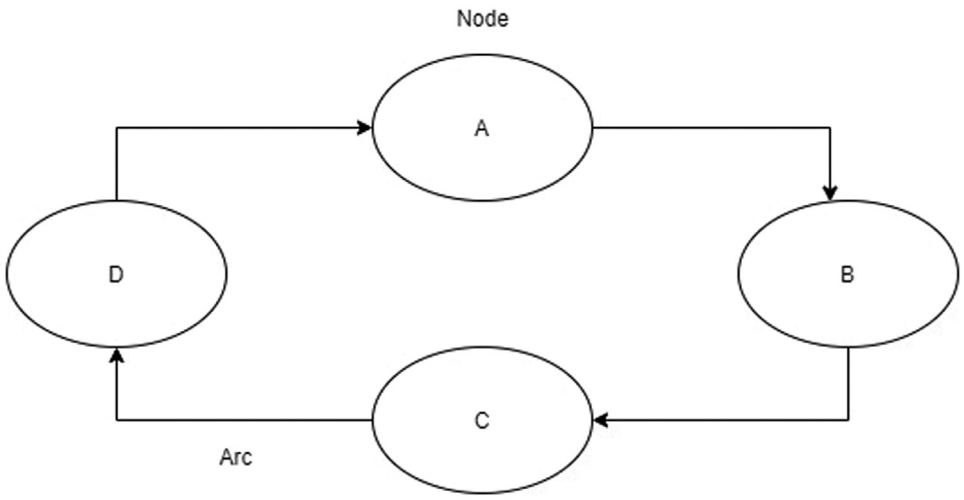

FIGURE 27.3 Bayesian belief network representation.

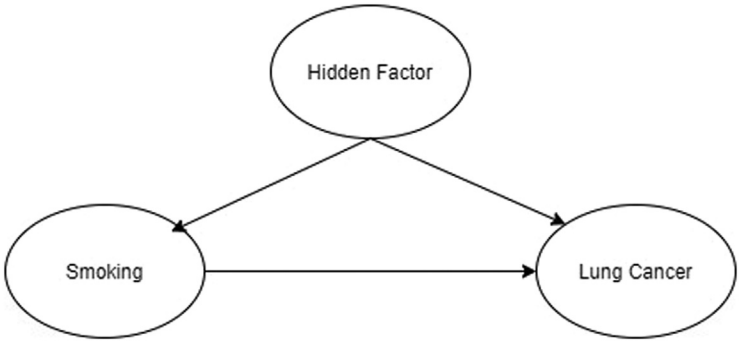

FIGURE 27.4 A conditional causal model example.

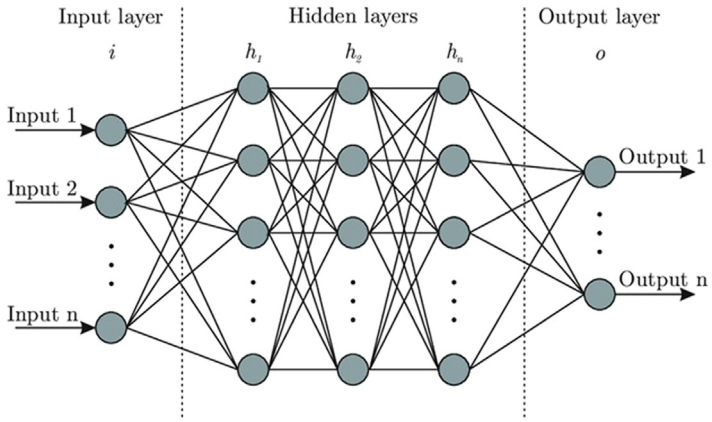

FIGURE 27.5 Neural network layers.

27.4.5 Neural network

A neural network is a model comprising layers of exceptionally interconnected handling units that can be prepared to perform arrangement undertakings. Examples of information and yield are first introduced to the model for preparing. The sub-symbolic knowledge of a prepared model is certainly put away in multiple associations or bolts highlighting and away from the inner portrayal units. Different techniques exist to prepare a neural network; the most often utilized is back-engendering with the summed-updelta rule. With back-spread, the client characterizes the number of shrouded layers and hubs in each layer. At that point, the model produces an original yield, in light of random loads of the associations. This yield is contrasted and the ideal yield and the distinction between model forecast and wanted yield is calculated (Wall & Brotherstone, 2005). The absolute squared aggregate of the determined contrast is then returned into the model, and loads of the associations are changed to limit the mistake. This method is rehashed commonly for all mixes of information and yield as shown in Fig. 27.5.

A definitive objective for the model is to locate a solitary arrangement of loads that fulfill all the sets of information and yield introduced to it, which is summed up to characterize new data accurately. Neural networks are acceptable as a design acknowledgment; they require no presumptions on data recurrence or dispersion, and, in the wake of preparation, neural networks can perform arrangement undertakings with missing data. Consequently, prepared neural networks can work with deficient declarative knowledge.

27.5 Application of machine learning in dairy industry

From a recognized and misjudged history to the current day, with the utilization of various natively created methods, and the expanding use of adjusted advances from the global examination, smallholder dairying in the jungle has set up a suitable and expanding future. The utilization of information has been pushed by many; they gave decency to a field which during the 2000s should grow. All things considered, in connecting social and regular researchers on the side of additional advancement of smallholder dairying in the jungles, a more extensive understanding by researchers and

instructors are required. Researchers should understand the constant connection between selection, new innovation advancement, and socio-social prerequisites, and little ranchers who have customarily been disregarded and who need schooling about the connections between different species. Similarly, as with numerous parts of under-developed nations' horticulture and incorporated cultivating frameworks, it is hard to characterize a person as a smallholder dairy rancher alone as this might be basically one of the numerous occupations (Weigel, 2004). The worldview utilized in our investigation of such endeavors is in itself a constraint to our capacity to additionally improve perplexing, productive, incorporated frameworks. The various tests for improving offices, researchers, and teachers is one of expanding their own insight into the factors and connections, such as exceptionally incorporated frameworks that include the focal part of the smallholder rancher within them.

27.5.1 Application of machine learning in milk procurement and billing

Investigation of information for the amounts of milk providing fixates dependent on quality and amount was tedious employment before automated charging framework came into the dairy industry. One can envision getting milk in two movements from thousands of breeds that give milk. Added with these elements, are other complex installment conditions identified with acquiring milk that include estimating strategies, fat premise, twofold hub premise, complete solids premise, motivations for advancing milk obtainment amount and quality, negative motivators for controlling noncertifiable and low-quality milk, and so forth. Various people were needed in the manual framework to deal with this, which was tedious and less adaptable as well (Windig & Veerkamp, 2004). In any case, machine learning made it achievable with not much expense or time. Machine learning has demonstrated as an aid in overseeing colossal data identifying with milk assortment, quality checking, specialized sources of information, observing of managed impregnation exercises, and giving convenient installment to drain makers. Electronic data framework can assist with deciding milk acquirement costs and its effect on special costs. A fast examination of milk worth would be useful in the viable acquisition of milk and ration products.

Information about milk courses, limits of vehicles to collect milk, gathering timing, and quality status helps in planning. A bundle had been created by the computer center to produce bills for installment to ranchers occasionally for the supply of milk, dependent on its fat and SNF content. In spite of the fact that it didn't have a lot of pertinence to National Dairy Research Institute (NDRI) where acquirement of milk from ranchers isn't by and by, yet the bundle has its convenience in circumstances where milk is being obtained by the milk plants and installment is made at appropriate spans dependent on Solid not fat (SNF) and fat substance. The bundle could be appropriately altered to join some other boundary according to the necessity.

27.5.2 Application of machine learning in plant automation

Mechanization that is fully incorporated or part insightful is done to suit the dairy's prerequisite. Complete ML programmed plants utilize programmed tasks for all activities. As discussed earlier, information is added into the data for milk amount and quality. In light of the assortment and demand of the market for milk and milk items, the arranging is finished. Full computerization with online messages are saved for opportune checking. All data with respect to the supply of milk and milk items, receipt/dispatches, misfortunes, and so forth are known immediately to deal with the tasks. Electronic tasks can handle the item quality in a better manner. This sensor is utilized to gauge the cycle result. The sensor input is given to the regulator for the change of variables that are liable for quality property. They convert the homestead produce into items with wanted credits utilizing unit activity, for example, drying, vanishing, cooking, and so on. Cycle control is utilized to run these tasks efficiently to give safe items reliably (Yang et al., 1999).

A machine learning model is produced for precise control of milk temperature that is influenced by fouling. It can compute precisely the expansion in steam temperature needed for keeping the ideal milk cleansing temperature. The outcomes using steam control are contrasted, and the outcomes with no control and this system were found good for controlling the milk source temperature (Nema & Datta, 2005). Computer-supported calculation, being quick, encourages on-line observing of the quality. The methods utilized revolve around planar electromagnetic sensors working with radio recurrence excitation. The sensor innovation proposed can perform volumetric penetrative estimations to gauge properties all through the main part of the item (Luthria et al., 2006).

Utilization of dairy and food items can be followed back to earlier times. The dairy and food industry lingered behind other assembling businesses, for example, autos and petrochemical enterprises, in presenting robotization and mechanized cycle control. The dairy business is particularly situated for simple appropriation of mechanized cycle control since it requires broad recordkeeping, completed items by and large homogenous with generally a couple of fixings;

and liquid tasks are regularly of a long span, consecutive, and versatile to programming frameworks created for constant cycles. The high limit of current constant sanitization was conceived by the improvement of the sterile programmed redirection valve, utilized for dependable temperature detecting and similarly solid rationale control (at first with transfers, then with programmable rationale regulators).

Standardization of milk is the essential activity after receipt of milk in a dairy plant. In the event that the fat substance of incoming milk is known and is provided at consistent rate, it is adequate to quantify and control the progression of cream from the separator to acquire milk of a wanted fat substance. Standard control setup of a splash dryer incorporates two circles. In one circle, heat estimating delta temperature of hot air controls heat input. In the other circle, the feed rate is constrained by estimating the source temperature. Just as during the time spent making cheddar, controlled mixing of the curd during coagulation gives control of consistency, which is an element of ensnared air. From the arrangement tanks, curd streams at a controlled rate on to a whey expulsion transport. The measure of seepage is constrained by changing the speed of the transport (Narsaiah, 2005).

27.5.3 Application of machine learning in dairy computerized network

Associations are using advantages of networking by interfacing one division and/or association through electronic framework. More data and better checking is attainable with the assistance of wide zone organizing applications. National Dairy Development Board (NDDB) Anand has created mechanized systems administration frameworks of all the milk associations and alliances. The use of PC incorporates utilization of GIS, National Information Network (NIN) and so forth; computational neural network-based models have been effectively applied in different issues at the NDRI. The examination in this field is a work in progress across the globe. There has been little investigation into the use of computational neural networks in the field of horticulture and dairying, specifically particularly in India (Adesh et al., 2005).

Likely utilizations of models in dairy handling are quickly introduced:

1. Displaying of pH and acridity for cheddar creation has been made, utilizing computational neural network.
2. Time span of usability expectation of sanitized milk has been accomplished utilizing connectionist models.
3. Neural organizations have been effectively utilized to anticipate temperature, dampness and fat in chunk formed nourishments with palatable coatings during profound fat browning.
4. Model prescient control of an ultra-high temperature milk treatment plant has been acknowledged utilizing a neural framework.
5. The computational neural network procedure has been utilized to decide protein fixation in crude milk.
6. Investigation of dairy designs from an enormous natural information base has been performed utilizing neural organizations.
7. Neural organization models dependent on feed-forward back-engendering learning have been discovered helpful for expectation of dairy yield.
8. Computational neural network have been utilized for dairy yield forecast just as cow separating characterization.
9. Expectation of bovine execution with the connectionist model has demonstrated preferred outcomes over customary techniques.

27.5.4 Application of machine learning in dairy packaging

Automated framework or robots help to mitigate dreary, dull assignments for workers—all the while making the creation cycle more practical. Apparatuses for the pressing of dairy items regularly highlight a few capacities. The capacity relies upon the setup of the item being referred to. Apparatuses for pull, cutting, clasping or grasping might be utilized. It is likewise conceivable to join an assortment of devices that can be utilized to handle partition sheets, items, boxes, beds and so on It creates and supply total arrangements including marking, partition sheets, and palletizing items prepared for transportation.

The modernized framework utilized by Siemens Global Framework (Germany), remembers filling and bundling for plant, speak to significant expense factors on the way convey dairy bundling is prepared; these are outcomes from heterogeneous and detached arrangement. A productive option with advance bundling, the coordinated computerization answer for filling and bundling with machine. The Siemens upgraded bundling line coordinates filling and bundling frameworks in a typical mechanization and correspondence standard. The standardization and coordination of individual machines conveys cost-and energy-investment funds all through the creation line. Joining chances are lower and permit

the utilization of versatile creation information, securing and assessing frameworks from the enhanced bundling line like line overview, diagnostics, OEE, following and energy recording and executives. Moreover, the expenses of preparing, activating, and adjusting can be diminished fundamentally with an improved bundling line. Moreover, dairy bundling line effectiveness, efficiency, and accessibility during activity are altogether recognizably improved.

27.5.5 Application of machine learning in supply chain integration and traceability

Machine learning applications are likewise helping supply chains become all the more vertically coordinated. Better participation among ranchers and purchasers along the store network mitigates default hazard. Amul in India has introduced automatic milk collection unit systems in town dairy cooperatives. These frameworks upgrade the straightforwardness of exchanges between the rancher and the customer, and have brought down handling times and expenses. The application utilizes PCs associated with the internet at the milk assortment and focuses to report inventory network information, for example, fat substance and milk volumes secured (Bowonder et al., 2005). Dairy information service kiosks at assorted locations portray best practices in creature care to upgrade milk yield and quality and helps dairy cooperatives to successfully plan and coordinate veterinary, planned impregnation, steers feed, and related administrations (Bai et al., 2001). Conveyance of such exhaustive data assists with improving combination of the inventory network, subsequently decreasing default hazard. The early recognition of creation unpredictability makes it conceivable to take preemptive measures to address the hidden danger.

Machine learning applications have had an effect in alleviating two extra types of danger in the production network: clean and phytosanitary (SPS) danger, and default hazard. Bigger aggregators and brokers use programming frameworks to gather and track data about who is developing what, and whether ranchers are sticking to the sanitation and quality standards forced in Europe and North America, particularly for transient nourishments.

27.5.6 Application of machine learning in vendor development

The more normal arrangement of stock administration that is utilized related to an item date stepping framework is FIFO (First In, First Out). Utilizing FIFO, the item with the soonest termination date is specially positioned on the retail rack available to be purchased. With this framework, it is as yet conceivable to place ruined items before a client that aren't new to the taste, or perhaps not healthy or safe. This is on the grounds that the variety in the temperature history of some random item bundle is genuinely enormous, and some may actually terminate before the lapse date says they will. Accordingly, when misuse temperature conditions are experienced during stockpiling, transport, and handling, the FIFO strategy can't make up for the expanded crumbling, and the consistency in the nature of the item conveyed from the store is undermined.

An option in contrast to this is to decide the issue based on noticed or assessed food quality as opposed to expiration time. This is called Least-Shelf-Life, First Out (LSFO) or Shortest-Remaining Shelf-Life (SRSL) strategy. In this framework, if the temperature detecting and the combination capacity of the labels shows a previous sign in the tag (flagging a lower remaining time span of usability), at that point the item is returned to the retail rack. This rotation is absolutely free of the item dating. Under this situation, the chance of setting "awful item thought great" before the purchaser is nearly decreased to zero. This arrangement would decrease food squander and give more predictable quality at the hour of issue for food items which have been in contrasting temperature conditions.

PC is utilized in material administration work for stock control, report age, age of requests and seller execution appraisal. This guarantees financially savvy sourcing of value materials.

27.6 Dairy farm management functions

Like other management obligations, dairy farm management incorporates three key management capacities. These are Planning, Implementation, and Monitoring and Evaluation (Controlling). For hypothetical purposes, it might be advantageous to isolate the capacity of dairy farm management, yet essentially these capacities are covered in nature, for example, they are indivisible. Each capacity mixes into the other, and each influences the presentation of the others.

27.6.1 Planning

Planning is the essential capacity of dairy farm management. It is choosing ahead of time—what to do, when to do, and how to do it. Planning is deliberately contemplating ways and means for achievement of pre-decided objectives. It

overcomes any issues from where we are and where we need to be. The Planning capacity of dairy farm management incorporates the following segments.

27.6.1.1 Evaluating the inner and outside circumstance of the dairy farm

The arranging cycle begins with the evaluation. The evaluation takes a gander at the entire farm framework to distinguish and organize main points of interest, openings and choices for change. It may very well be finished utilizing investigation of farm execution accounts and benchmarking data, on-farm perception and an organized conversation with the dairy farm group or relatives occupied with the farm. The two most significant instruments that can be utilized to do dairy farm evaluation are issue/target examination and SWOT investigation.

27.6.1.2 Setting objective

Objective is a particular outcome that an individual, a framework or a business element might want to accomplish in a predefined period and with apportioned assets. Target setting is a significant cycle in the arranging cycle. Goals fill in as a reason for making normal understanding among various partners on what is relied upon to be accomplished and to contrast execution and plan. In dairy farm management, setting a target should be possible for the endeavor/dairy farm/all in all (for a model producing a specific measure of pay), yet additionally for explicit segments of the farm. That incorporates fodder creation, protection, stockpiling and feed flexibly, feeding management, milking and milk creation, youthful stock raising for supplanting winnowed and sold dairy bovines, breeding and fertility management, health management, and housing and fertilizer management. Destinations ought to likewise satisfy the SMART criteria (Specific, Measurable, Achievable, Realistic and Time bound).

27.6.1.3 Strategy design

When we set targets for the farm, the subsequent stage in the arranging cycle is planning a methodology to understand those goals. The technique is the strategy or approach picked to bring the accomplishment of the ideal outcomes or goals.

27.6.1.4 Activity design and resource planning

This is the final step in the planning process. Here we develop specific operational activities the farm will undertake to achieve its objectives, along with the implementation timeframe, required resource, and responsible person. The resource planning will identify:

1. What is required?
2. How much is required?
3. From where the required resource will be obtained (source)?

27.6.2 Implementation

Usage is the execution of arranged exercises according to the timetable and apportion assets to bring the expected outcomes. Execution work incorporates the accompanying key duties:

1. Satisfying the fundamental human and non-HR needed to understand the farm targets.
2. The assets could be prepared inside or from an outside source.
3. Sorting out and driving the farm human asset in a proficient and compelling manner.

27.6.3 Monitoring and evaluation

Assessment and control capacity of dairy farm management incorporates three key advances: estimating execution, correlation of plan vs accomplishment, and making remedial moves. Assessment and control measures are regularly joined in dairy farm management. They are consolidated in light of the fact that they are entwined measures in which the farm proprietor asks how it is getting along, and what does it have to change to be effective. The essential inquiry to be posed in every one of the cycles is introduced next.

27.6.3.1 Assessment

The assessment cycle askes the accompanying three key inquiries:

1. Where are we now in examination with where we need to be (plan)?
2. What lies ahead that can influence the farm either emphatically or adversely?
3. Where will we end up in the event that we proceed on the current way?

Responding to these inquiries is what we mean by assessment. The cycle gives what we call "gap examination."

27.6.3.2 Control

After the farm proprietor has assessed its exhibition, he should address the inquiry, "What changes should be made, how and where?" The decision to make changes or not is the start of the control cycle.

27.7 Future perspective

The eventual fate of smallholder dairying in the jungles will be portrayed by various interesting elements. These will be progressively perceived as qualities of significance in their own privilege as opposed to varieties from an attractive standard in dairy creation, as may right now are in the situation. Such qualities would include:

1. Creation of milk as one of numerous yields from coordinated cultivating frameworks;
2. Dependence on smallholder dairying for most of the local region milk and milk item supply;
3. Expanded concentration for easier new milk creation for local towns;
4. Creation of boutique milk items, arranged to local tastes;
5. Usage of waste and side-effects as guidelines to take care of creatures;
6. Transformation to and use of accessible local data sources;
7. Improvement dependent on self-improvement, driving now and again to collectively claimed preparing offices;
8. Enhancing instead of amplifying milk creation inside a minimal-effort creation framework;
9. Public farming exploration framework interest in smallholder dairying research; and
10. Links among rustic and metropolitan regions through arrangements of movable nutritious and, in a protected structure, a durable item.

27.8 Conclusion

Machine learning will upgrade arrangements in the dairy industry. Utilizing the arrangement, the client has advanced their creation by diminishing creation and unit costs. Information technology/mechanization will improve the actual workplace, considering the quantity of dreary, tedious undertakings to be dispensed with or limit, expanding productivity will be underway. Machine learning accessible in farming today makes it conceivable to deal with a dairy industry on a more itemized level than previous. The dairy administrator can settle on a more levelheaded choice by getting the measure of data; the dairy director needs to work a few PCs every day and physically move information starting with one unit then onto the next. The chapter has broken down data possibility and the use of machine learning in the present-day dairy industry. This framework as the dairy board instruments portray, archive, and control all cycles on dairy creation, particularly the multi-reason and multi-specialist framework application that uphold the executives of the dairy and give documentation to all network individuals. Customization of IT stages for use in the dairy industry is arising as a significant open door for change. As machine learning frameworks become instrumental in accommodating the wellbeing of dairy items, it confirms that appropriate controls are utilized to guarantee precise, predictable, and solid outcomes gotten from PC control and information stockpiling frameworks.

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