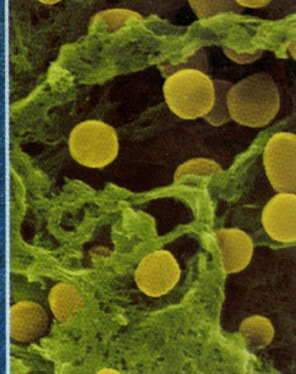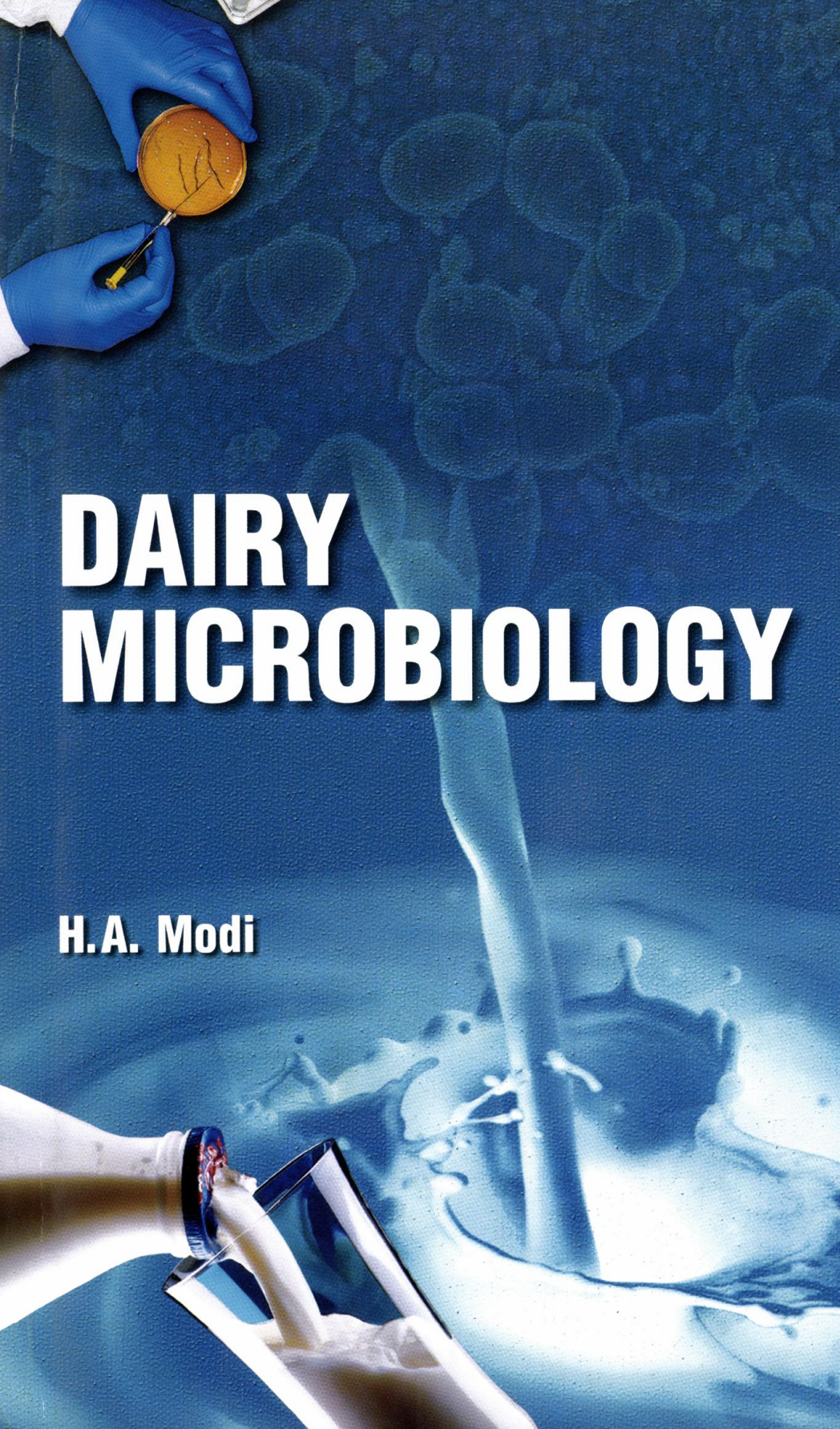# DAIRY MICROBIOLOGY

H. A. Modi

DAIRY MICROBIOLOGY

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Dairy Microbiology

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PREFACE

Chiefly, as a result of White Revolution, India is now aiming to reach the status of World Number one milk producer in the world. In this context, especially in the post-GATT scenario, it has become imperative for Indian Dairy Industry to adopt cost-effective state of art cutting edge technologies to produce International Quality Dairy Products.

Dairy development scenario all around the world has shown steady rise in respect of both increase in milk production and manufacture as well as consumption of dairy products. However, India has exhibited unprecedented rate of growth due to "Operation Flood Programme" introduced by National Dairy Development Board [NDDB].

A professional in dairy field is incomplete without the fundamental knowledge of Dairy Microbiology, which covers the important area of quality management of raw materials and finished products and help in checking the processing efficiency. It also helps in dairy product manufacture through use of selected microorganisms (as starter cultures) and prevent the entry of harmful pathogens to the products, thereby proving the need and importance of Dairy Microbiologists.

The present **"Dairy Microbiology"** book attempts to offer basic concept of all the essential components of Dairy Microbiology and should prove of help to all students and professionals engaged in dairy activity and bussiness. This compilation will prove of great help to students pursuing under-graduate or post-graduate courses in Dairy Science and Technology, Food Science and Technology, Agriculture and Veterinary Science, Home Sciences and Home Economics as well as other basic science courses (Microbiology, Biotechnology, Biochemistry, Environmental science, and Food Nutrition) where this subject forms a part of its applied microbiology curricula. Compactness and simplicity in presenting the subjective information will make the book of great value to students having first exposure to the subject.

The book includes twenty-four chapters and four appendices. The first chapter introduces Milk and Dairy Microbiology subject. Microbiology of raw milk covering sources

of microorganisms in milk, micro flora, raw milk testing etc. are discussed in chapter 2. Milk-borne diseases are summarized in chapter 3. Principles and method of pasteurization and sterilization of milk are explained in chapter 4. Grading of milk as well as sanitization of dairy plants is also discussed in same chapter 4. Processing and microbiology of "Concentrated Milks" like sweetened condensed milk and evaporated milks are discussed in chapter 5. Similarly, processing and microbiology of "Dried Milks" (milk powder) and dry whey products are elaborately explained in chapter 6. Starter cultures are selected groups of pure and actively growing microorganisms, which are used singly or in combination of inoculum (intentionally added) to bring about desirable changes in milk to form required products. Starter cultures have a multifunctional role in dairy fermentation industry. The Microbiology (chapter 7), Metabolism (chapter 8), and Genetics (chapter 9) of starter cultures are discussed in depth in this book. Various microbiological aspects like nature, desirable characteristics, classification, propagation, problems etc. of starter cultures are discussed in chapter 7.

Fermented milks possess many advantages like easy production, better keeping quality and nutritive value, easier digestibility, pleasing flavour and tastes and strong therapeutic potentials and so they are more popular nowadays. Various microbiological aspects of "Fermented Milks" are discussed in chapter 10. A variety of fermented milks (about one and half dozen) right from "Acidophilus Milk" to "Sri khand" are commerically produced. Definition, manufacturing methods, nutritive and therapeutic value, microbiology of each fermented milk is described in chapter 11. Cheese is a very valuable and popular dairy product manufactured from milk. Various microbiological aspects of cheese production are discussed in chapter 12. Commercial manufacture of about a dozen type of cheese are schematically explained in chapter 13, taking examples like Quark, Cottage Cheese, Harzer Cheese, Swiss Cheese, Cheddar Cheese, Limburg Cheese, Camembert Cheese, Roquefort Cheese (blue), Edam and Gouda, Italian mozzarella Cheese, Process Cheese etc. The processing and microbiology of "Ice cream and related Dairy Desserts" are described in chapter 14. Manufacturing and microbiological aspects of "Butter and related products like Margarines and Dairy Spreads" are discussed in chapter 15.

Dairy byproducts like "Whey, Casein and Caseinates" and their utilization are discussed in chapter 16. Probiotics are viable bacterial cell preparation of milk/food containing useful bacteria that have beneficial effects on the health of consumer. Various characteristics and heath benefits of such "Probiotics" are explained in chapter 17. Physio-chemical testing of milk and dairy products are described in chapter 18, while routine microbiological examination tests are explained in chapter 19. Microbiological standards for maintaining quality of different dairy products are also summarized in chapter 19. Composition of microbiological tests in dairy industry area are also mentioned in the same chapter 19. The chapter 20 briefly explains the implementation of a "HACCP program", and provides a model HACCP program as a guide to develop an effective system in a dairy plant, by taking an example of "Bottled Milk". "Shelf-life predicting methods for milk" are described in chapter 21. "Indigenous (Indian) Dairy products" like Khoa, Basundi, Kheer, Panner, Chhanna, etc. are described in chapter 22. "Microbial control by new non-thermal methods" are described in chapter 23. Dairy industry produces a large amount of waste-water or effluent. "Dairy Waste and Treatment" are briefly summarized in chapter 24, by

giving an outline design for 2,00,000 liters/day capacity effluent treatment plant for milk dairy.

In addition to above information-rich twenty four chapter, an additional four appendices are included in the book for benefits of readers : (1) Taxonomic characteristics of Starter Cultures (Appendix-A), (2) Differential staining Techniques, mentioning Gram staining and Acid fast staining (Appendix-B), (3) Enzymes (Lactase, Beta-galactosidase, Proteases, Lipases) used in dairy industry (Appendix-C) and (4) Explanation of important terms used in Dairy Industry as a short glossary (Appendix-D).

A fully referenced text is not, in my view, appropriate to a book of this type aimed at readers with little or any knowledge of this subject. However, a list of selected bibliography is included, to assist students in expending their knowledge on the subject. The materials in each chapter are arranged in logical, systematic and concise sequences. Tables, Figures and data have been used when their presentation is necesary for better understanding.

No book can be completed without the dedicated help of many individuals. Especially, I would like to express my eternal gratitude to Principal (Retd.) B.N. Gandhi for his inspiration, moral support, and valuable help. I would like to thank all members of my faimly for "putting up" with me during the preparation of this book. Those hours that I spent working on the book were hours during which I neglected them.

I am grateful to Aavishkar Publishers, Distributors, Jaipur for their concern, efforts and encouragement, especially for their excellent cooperation in the task of preparing and publishing this book.

—**H.A. Modi**

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CONTENTS

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INTRODUCTION TO MILK AND DAIRY MICROBIOLOGY

1.1 INTRODUCTION

Prebiotics (non digestable components of food) and probiotics (live microbial feed supplement that enhance the host health by modulating the intestinal microbial balance) or synbiotics (combination of pre-and pro-biotics) alter physical, microbial and chemical composition of intestinal micro-environment and have the potential to improve the health status of the consumers. In recent years, Probiotics/Synbiotics have revealed great potential in alleviating several gastrointestinal disorders, pre/post-operative infections, colorectal and certain cancers and have also shown potential in treatment of even AIDS.

Functional foods are foods that give therapeutic benefits to hosts in addition to nutrition. Dairy foods and ingredients in general and fermented dairy products in particular are well recognized for their nutritional and therapeutic attributes and as functional foods.

The public interest is greater than ever towards consuming something natural with health benefits that can enhance the human welfare and at the same time reduce the use of medicines especially antibiotics. This has changed the market dynamics and the functional food market, which is expected to triple in next five year world over. Indian food industry will also have ample opportunities in coming years on development of functional dairy products.

Although the art of preparing cultured dairy foods, such as cheese, yoghurt, kefir and koumiss, has been practised for ages, their science and technology as practised today are of modern origin. Advances made in sciences of microbiology, biochemistry and engineering, particularly during the past two or three decades, coupled with the progress achieved in industrial microbial technology, have led the way for the isolation and identification of more specific cultures and for the development of newer and a wider variety of cultured dairy products.

1.2 HISTORICAL SIGNIFICANCE

Milk has been used as food since antiquity although it is not known when prehistoric man actually domesticated the cow, sheep or goat for milk production. Archaeologists have found evidence in the Libyan desert that cows were used for milk as far back as 9000 BC (Pederson 1979). Fermentation occurred naturally in surplus milk set aside for later use, and this process served as a primitive method of food preservation. It was no doubt observed that in some cases the milk formed a smooth curd with a pleasing acid odour and flavour, while in other cases the milk became putrid and caused illness when consumed. The preparation of fermented, or cultured, milk products then became an art which was handed down from one generation to another.

Milk is a complex mixture consisting of an oil-in-water emulsion stabilized by phospholipids and protein adsorbed on the surface of the fat globules. It also contains the water-soluble B-vitamins (thiamin, riboflavin, pyridoxine, niacin, pantothenic acid, biotin and folic acid), vitamin C, the fat-soluble vitamins (A, D, E and K), salts, citrates, enzymes (lipase, catalase and phosphatase) and the carbohydrate lactose—all the requirements for the growth of the fastidious lactic acid bacteria (Pederson 1979).

In raw milk, the types of organisms which develop depend on the introduction of organisms into the milk, sanitary conditions, prevailing temperatures and other environmental factors. According to Pederson (1979), the lactic organisms most likely to grow first in milk will be *Streptococcus lactis* and *Strep. cremoris*; in warm climates, however, *Strep. thermophilus* will be the first to predominate. These organisms produce sufficient acid to prevent the growth of acid-intolerant spoilage organisms and to promote the growth of the more acid-tolerant lactic bacteria such as *Lactobacillus bulgaricus* and *L. casei*.

Milk of a variety of animal species, fermented by lactic cultures, is known by different names in different parts of the world. Though all the products are produced commercially, the best known of these in the Western world are yoghurt, acidophilus milk, kefir and koumiss. Few people recognize, however, that these products are prepared by bacterial and/or yeast action and that the characteristic flavours and textures of these products are results of these fermentations.

According to legend, the method of preparing yoghurt was revealed to Abraham by an angel, and he owed his fecundity and longevity to this fermented food (Rosell 1932). Another cultured product of similar and ancient mysterious origin is kefir, which is also known as the 'champagne of milk' and the 'drink of the prophet'. In Russia, Tolstoy conscientiously consumed koumiss for treatment of his tuberculosis (Tolstoy 1971), while Metchnikoff (1908) proposed that the longevity of the Bulgarians was due in part to their ingestion of large quantities of bulgarican milk. Metchnikoff was perhaps the first person, however, to associate the action of specific microorganisms with the nutritional and therapeutic properties of cultured products.

1.3 NEED OF DAIRY MICROBIOLOGY AND DAIRY PERSONNELS

Chiefly, as a result of White Revolution, India is now aiming to reach the status of World Number one milk producer in the World. In this context, especially in the post-GATT scenario, it has become imperative for the Indian Dairy Industry to adopt cost-effective state of art cutting edge technologies to produce International Quality Fermented Products.

Dairy development scenario all around the world has shown steady rise in respect of both increase in milk production and manufacture as well as consumption (Table 1.1) of dairy products. However, India has exhibited unprecedented rate of growth due to Operation Flood Programme introduced by National Dairy Development Board (NDDB). During the last 25 years, the milk production in the country has increased by almost 2.5 to 3 times, and the achieved figure for the year 2000 A.D. is more than 78 million tonnes. The milk processing capacity of 11.7 million liters per day in 1987 is achieved more than 27.5 million liters per day in 2000 A.D.

Table 1.1 : Per capita consumption (kg) of milk and milk products in various countries (Candian Dairy Information Centre, 2000)

Country	Fluid Milk	Cheese	Butter
Australia	102	11.1	3.2
Canada	90	11.6	2.6
France	68	23.2	8.8
Germany	63	12.8	6.7
India	33		1.9
Ireland	163	07.0	4.0
Italy	63	20.6	1.7
Japan	39	1.8	0.6
Netherlands	101	14.6	3.1
Sweden	157	16.6	1.5
U.K.	117	0.7	3.0
U.S.A.	97	14.0	2.0

To meet the challenge of handling huge quantity of perishable milk efficiently, a well-planned approach implemented with the help of properly trained professionals is required. The training has got to be multi-pronged to develop skills and abilities to cope up with milk collection job from distant places under difficult conditions, efficiently processing it and converting into varieties of high tech value-added products adopting appropriate technologies from home and abroad, and achieve required level of quality matching with the demand of competitive domestic and foreign markets.

A professional in dairy field is incomplete without the fundamental knowledge of Dairy Microbiology, which covers the important areas of quality management of raw materials and finished products and helps in checking the processing efficiency. It also helps in dairy product manufacture through use of selected microorganisms and prevent the entry of harmful pathogens to the products.

1.4 DEFINITION OF MILK

Milk is the liquid food secreted by the mammalians for the nourishment of their new borns. However, since ancient times, humans have exploited this natural food for their own benefits.

The utilization of milk has become a business and hence it is required to be legally defined. Each country sets its own standards for major constituents of milk and allow its safe trade.

In India, Prevention of Food Adulteration (PFA) Act defines most of the food commodities with certain standards. According to PFA, milk is defined as "a secretion derived from complete milking of healthy milch animals. It shall be free from colostrums. Milk of different classes and of different designations shall conform to the standards prescribed in the Act." (Table 1.2)

Milk can also be defined based on its chemical make-up. Hence, chemically speaking "milk is a substance in which fat is present in emulsion, casein (major milk protein) together with some minerals in colloidal suspension while lactose, mineral salts and whey proteins in watery solution."

Table 1.2 : Standards of different types of milk as per PFA Act in India

Class of milk*	Minimum (%)	
	Milk fat	Milk SNF
1. Buffalo milk	5.0 to 6.0	9.0
2. Cow milk	3.0 to 4.0	8.5 to 9.0
3. Goat/sheep milk	3.0	9.0
4. Mixed milk	4.5	8.5
5. Standardized milk	4.5	8.5
6. Recombined milk	3.0	8.5
7. Toned milk	3.0	8.5
8. Double toned milk	1.5	9.5
9. Skim milk	0.5	8.7

*Note—When milk is offered for sale without indication of the class, the standards prescribed for buffalo milk shall apply.

1.5 TYPES OF MILK

Milk is generally designated on the basis of the species of animal from which it is drawn, e.g. cow milk, buffalo milk, goat milk, human milk etc. However, for trade, milk may be treated or modified in different ways and may be designated in different ways and may be designated by different names.

(1) Raw Milk—Milk is the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which contains not less than 8 ¼ percent milk solids-not-fat and not less than 3¼ percent milk fat. Milk drawn from udders in native state is called raw milk.

The food value of milk depends upon its milk fat and its solids-not-fat content. If either of these is reduced below the range for normal market milk, the proteins, carbohydrates, minerals, and certain vitamins are also reduced. Practical experience shows that 3 ¼ percent fat and 8¼ percent solids-not-fat are reasonable minima for mixed-herd milk.

Milk produced within 15 days before calving or 5 days after calving is likely to contain colostrum. Colostrum tends to produce intestinal disturbance in children.

(2) Goat milk—Goat milk is the lacteal secretion, practically free from colostrum, obtained by the complete milking of healthy goats. The word "milk" shall be interpreted to include goat milk.

(3) Mixed milk—A combination of milk from different species of animals.

(4) Cream—Cream is the sweet, fatty liquid separated from milk, with or without the addition of milk or skim milk, which contains not less than 18 percent milk fat.

(5) Light cream, coffee cream, or table cream—Light cream, coffee cream, or table cream is cream which contains not less than 18 percent but less than 30 percent milk fat.

(6) Whipping cream—Whipping cream is cream which contains not less than 30 percent milk fat.

(7) Light whipping cream—Light whipping cream is cream which contains not less than 30 percent but less than 36 percent milk fat.

(8) Heavy cream or heavy whipping cream—Heavy cream or heavy whipping cream is cream which contains not less than 36 percent milk fat.

(9) Whipped cream—Whipped cream is whipping cream into which air or gas has been incorporated.

(10) Whipped light cream, coffee cream or table cream—Whipped light cream, coffee cream, or table cream is light cream, coffee cream, or table cream into which air or gas has been incorporated.

(11) Sour cream or cultured sour cream—Sour cream or cultured sour cream is a fluid or semifluid cream resulting from the souring, by lactic acid producing bacteria or similar culture, of pasteurized cream, which contains not less than 0.20 percent acidity expressed as lactic acid.

(12) Half-and-half—Half-and-half is a product consisting of a mixture of milk and cream which contains not less than 10.5 percent milk fat.

(13) Sour half-and-half or cultured half-and-half—Sour half-and-half or cultured half-and-half is fluid or semifluid half-and-half derived from the souring, by lactic acid producing bacteria or similar culture, of pasteurized half-and-half, which contains not less than 0.2 percent acidity expressed as lactic acid.

(14) Recombined milk and milk products—Recombined milk and/or milk products shall mean milk or milk products defined in this section which result from the recombining of milk constituents with potable water.

(15) Reconstituted milk—It is the milk prepared by dissolving dried milk powder in appropriate quantity of potable water.

(16) Concentrated milk—Concentrated milk is a fluid product, unsterilized and unsweetened, resulting from the removal of a considerable portion of the water from milk, which, when combined with potable water, results in a product conforming with the standards of milk fat and solids-not-fat of milk as defined above.

(17) Concentrated milk products—Concentrated milk products shall be taken to mean and to include homogenized concentrated milk, vitamin D concentrated milk, concentrated skim milk, fortified concentrated skim milk, concentrated low fat milk, fortified concentrated low fat milk, concentrated flavoured milk, concentrated flavoured milk products, and similar concentrated products made from concentrated milk or concentrated skim milk, and which, when combined with potable water in accordance with instructions printed on the container, conform with the definitions of the corresponding milk products in this section.

(18) Frozen milk concentrate—Frozen milk concentrate is a frozen milk product with a composition of milk fat and milk solids-not-fat in such proportions that when a given volume of concentrate is mixed with a given volume of water the reconstituted product conforms to the milk fat and milk solids-not-fat requirements of whole milk. In the manufacturing process, water may be used to adjust the primary concentrate to the final desired concentration. The adjusted primary concentrate is pasteurized, packaged, and immediately frozen. This product is stored, transported, and sold in the frozen state.

(19) Skim milk or skimmed milk—Skim milk or skimmed milk is milk from which sufficient milk fat has been removed to reduce its milk fat content to less than 0.50 percent.

(20) Low-fat milk—Low-fat milk is milk from which a sufficient portion of milk fat has been removed to reduce its milk fat content to not less than 0.50 percent and not more than 2.0 percent.

(21) Vitamin D milk and milk products—Vitamin D milk and milk products are milk and milk products, the vitamin D content of which has been increased by an approved method to at least 400 U.S.P. units per quart.

(22) Fortified milk and milk products—Fortified milk and milk products are milk and milk products other than vitamin D milk and milk products, the vitamin and/or mineral content of which have been increased by a method and in the amount approved by the health authority.

(23) Homogenized milk—Homogenized milk is milk which has been treated to ensure break-up of the fat globules to such an extent that, after 48 hours of quiescent storage at 45°F, no visible cream separation occurs on the milk, and the fat percentage of the top 100 ml of milk in a quart, or of proportionate volumes in containers of other sizes, does not differ by more than 10 percent from the fat percentage of the remaining milk as determined after thorough mixing. The word "milk" shall be interpreted to include homogenized milk.

(24) Flavoured milk or milk products—Flavoured milk or milk products shall mean milk and milk products as defined in this Ordinance to which has been added a flavour and/or sweetener, as well as with nuts, coffee, chocolate etc.

(25) Eggnog-flavoured milk—Eggnog-flavoured milk is a milk product consisting of a mixture of at least 3.25 percent butterfat, at least 0.5 percent egg yolk solids, sweetener, and flavouring. Emulsifier and a maximum of 0.5 percent stabilizer may be added.

(26) Eggnog—Eggnog is a milk product consisting of a mixture of milk or milk product of at least 6.0 percent butterfat, at least 1 .0 percent egg yolk solids, sweetener, and flavouring. Emulsifier and not over 0.5 percent stabilizer may be added.

(27) Buttermilk—Buttermilk is a fluid product resulting from the manufacture of butter from milk or cream. It contains not less than 8 ¼ percent of milk solids-not-fat.

(28) Cultured buttermilk—Cultured buttermilk is a fluid product resulting from the souring, by lactic acid producing bacteria or similar culture, of pasteurized skim milk or pasteurized low-fat milk.

(29) Cultured milk or cultured whole milk buttermilk—Cultured milk or cultured whole milk buttermilk is a fluid product resulting from the souring, by lactic acid producing bacteria or similar culture, of pasteurized milk.

(30) Acidified milk and milk products—Acidified milk and milk products are milk and milk products obtained by the addition of food grade acids to pasteurized cream, half-

and-half, milk, low-fat milk, or skim milk, resulting in a product acidity of not less than 0.20 percent expressed as lactic acid.

(31) Standardized milk—Milk solids, especially milk fat is very costly. Milk price is also decided based on its fat and SNF (solid-not-fat) content. Hence, for cost benefits and for providing uniform quality milk to the customers, the dairies standardize the milk with respect to fat and SNF content at specific level before sale. Such adjusted milk is called standardized milk.

(32) Toned milk and Double toned milk—These milks are types of standardized milk but adjusted to low fat levels to make them relatively cheap.

1.6 MILK CLASSIFICATION

From the microbiological points of view, the milk can be classified into three categories—

(A) Fresh milk—This milk is endowed with all the naturally occurring constituents provided the milking is done perfectly asceptically and the milking cattle are healthy. This milk has great nutritional value. In Ayurveda this milk has been referred to as *Dharoshna* milk meaning thereby that the freshly drawn milk has the same temperature as the body of the milking cattle. In this milk no constituent is lost and destroyed. But, use of this milk has its own limitations.

(B) Pasteurized milk—This milk is treated at a definite temperature for a definite time period so as to get it free from the microorganisms without losing any constituent. This treatment is called **pasteurization** which results in the destruction of most of the diseases and spoilage causing microorganisms in milk with heat.

Actually, the term 'pasteurization' refers to the process of heating every particle of milk to at least 145°F and holding it continuously at or above this temperature for at least 30 minutes, or to at least 161°F or above this temperature for at least 15 seconds. The process of pasteurization is a method of **'partial sterilization'** and was first introduced by L. Pasteur in wine and beer industry.

Home pasteurization of milk—Simplest and one of the most satisfactory methods of home pasteurization of milk is as follows—

The milk is usually pasteurized in regular milk bottles. In this process, cover from one bottle is removed, a little of the milk is poured out, a hole is punched in the cover, the cover is replaced and a thermometer is inserted there. Then all the bottles of milk are set on a milk rack and heated until the thermometer registers 145°F. The pail is removed from the heat and the bottles are left in the hot water for 30 minutes; reheating is done to keep the temperature at 145°F, if necessary. After the 30 minutes period, the hot water is gradually replaced with cold water until the milk is cooled, preferably using ice in the last water.

Commercial pasteurization of milk—Two methods of milk pasteurization are used commercially—

(i) Low temperature holding (LTH) method.

(ii) High temperature short-time (HTST) method.

(i) Low Temperature Holding (LTH) Method—In low temperature holding method, or 'vat pasteurization', the milk is heated to 145°F (62.8°C) for 30 minutes. The milk is heated in closed vats by steam coils, hot water jackets or continuous spraying of heated water along

the sides of the container. It is then cooled and bottled. It has been found in practice that LTH method is the more efficient as it destroys a larger percentage of the bacteria. Usually over 99% of the pathogenic bacteria present in milk are destroyed by proper pasteurization by this method.

(ii) **High Temperature Short-time (HTST) Method**—In this method milk is heated to a temperature of 161°F (71.7°C) for 15-30 seconds. The heating is done by means of electricity or hot water. The heated milk is then cooled and maintained at a low temperature until distributed.

(C) **Fermented Milk**—Fermentation is used to a very large extent with dairy area. Here the purpose is souring and developing flavour substances. Fermentation is done commonly of the pasteurized milk. The "souring of sweet milk" was practiced long before the microorganisms had been discovered. This was done to preserve milk and to provide a new beverage with a distinctive and desirable flavour. Consumption of fermented milk is widespread due to its therapeutic value. The most important organisms used in the preparation of fermented milk are species of *Streptococcus*, *Leuconostoc*, and *Lactobacillus*. The starter culture consists of either pure strain of a species or combination of species best suited for the production of desired product.

(D) **Sterilized Milk**—The milk heated to a temperature of 115°C for 15 minutes or 145°C for 3 seconds or equivalent approved time-temperature combinations is called sterilized milk. It should be sold only in the container in which it is sterilized and should ensure preservation for at least 15 days at room temperature.

1.7 COMPOSITION OF MILK

Milk is the normal secretion of the mammary glands of mammals. It is the "most nearly complete" food for humans, and is an excellent culture medium for many microbial species. Freshly drawn milk varies in chemical composition. Some of the more important causes of variation are the species of mammal, the breed, the age of the individual, the state of her lactation, her feed, her health, and the season of the year. The average chemical composition based on the analysis of a large number of milk samples is shown in Table 1.3.

Table 1.3 : Average composition (%) of cow, buffalo and human milk

Constituent	Cow	Buffalo	Human
Water	87.20	82.76	87.43
Fat	3.7	7.38	3.75
Protein	3.5	3.6	1.63
Lactose	4.9	5.48	6.98
Ash	0.70	0.78	0.21
Total Solids	12.80	17.24	12.57

Milk also contains several other minor constituents. Among these, salts (Ca, PO_4, Cl, Na, Mg, K, S, Fe) and trace elements are very important from physiological point of view. It also contains vitamins A, B_1, B_2, B_6, B_{12}, C, D, K. As well as, milk contains several enzymes, non-protein nitrogenous substances, pigments, etc. Milk is very complex mixture of all these compounds, that nature has given to us.

Table 1.4 shows detailed composition of cow's milk expressed as mean values in 100 gram of edible component.

Table 1.4 : Approximate composition of cows' milk

(A)	Water	*ca*, 87% (w/v)
(B)	Carbohydrates	*ca*, 4.9% (w/v)
		α-lactose 37% of total lactose
		β-lactose 63%
		(minor) Glucose, galactose, polysaccharides, sugar phosphates
(C)	Lipids	*ca*, 3.7% (w/v)
		Triglycerides 98% of total lipid
		(minor) Diglycerides, monoglycerides, phospholipids, sterols, carotenoids, vitamins A, D, E, K
(D)	Proteins	*ca*, 3.5% (w/v)

Casein	% of total protein
α-Casein	53-70
β-Casein	25-35
κ-Casein	8-15
γ-Casein	3-7
Non-casein	14-24
β-Lactoglobulin	7-12
α-Lactalbumin	2-5
Blood serum albumin	0.7-1.3
Euglobulin	0.8-1.7
Pseudoglobulin	0.6-1.4
Immunoglobulins	1.3-2.8
Proteose-peptone	2-6

(minor) Lipases, esterases, alkaline phosphatase, acid phosphatase, xanthine oxidase, lactoperoxidase, proteases, catalase, sulphydryl oxidase, amylase, aldolase, ribonuclease, carbonic anhydrase, saldase, rhodonase, lactase, lactoferrin.

(E)	Crude fiber	-
(F)	Minerals	0.74 g
	Potassium	157 mg
	Calcium	120 mg
	Iron	0.046 mg
	Phosphorus	92 mg
(G)	Vitamins	
	Vitamin A	30 µg

... contd.

	Carotene	18 µg
	Vitamin D_2	0.063 µg
	Vitamin E	88 µg
	Vitamin K	17 µg
	Vitamin B_1	37 µg
	Vitamin B_2	0.18 µg
	Nicotinamide	90 µg
	Pantothenic acid	0.35 µg
	Vitamin B_6	46 µg
	Biotin	3.5 µg
	Folic acid	-
	Vitamin B_{12}	0.42 µg
	Vitamin C	1.7 mg
(H)	Ash	0.7 (w/v)

Approximate chemical composition of milk from various milking animals are shown in Table 1.5.

Table 1.5 : The chemical composition of milk from various animals

Animal	Species	Composition, g/kg				
		Water	Fat	Casein	Whey protein	Lactose
Camel	*Camelus dromedarius*	865	40	27	9	50
Cow	*Bos taurus*	873	39	26	6	46
Goat	*Capra hircus*	867	45	26	6	43
Horse	*Equus caballus*	888	19	13	12	62
Sheep	*Ovis aries*	820	72	39	7	48
Water buffalo	*Bubalus bubalis*	828	74	32	6	48
Yak	*Bos grunniens*	827	65	—	58[a]	46

[a]Casein plus whey protein.

1.8 PHYSIO-CHEMICAL CHARACTERISTICS OF MILK

1. The white colour of milk is due to colloidal ca-caseinate and the greenish colour of milk whey is due to riboflavin. Upon heating whiteness increases due to increased reflection of light by coagulate. Carotenes and xanthophylls in leaves give a typical colour to cow's milk.

2. Fresh milk has an average pH of 6.6, corresponding to 0.16% lactic acid acidity. This is natural acidity of milk which is due to CO_2, H_2CO_3, citrates, proteins and acid phosphates and which gives a buffering capacity. Lactic bacteria also produce acidity.

3. Due to heating, the browning of milk occurs due to a complex formation between lactose and amino acids.

4. The characteristic taste of milk is due to lactose in it.

5. The skin formation upon heating is due to the protein lactalbumin, which breaks up forming fat globules.

6. Milk freezes at an average temperature of -0.55°C. The specific heat value of milk is 0.938 calories at 15°C. When shaken, milk foams due to trapped air, since the surface tension of the liquid is less. Due to this, it is preferable to purchase milk on a weight rather than a volume basis. Milk boils at 100.2°C. When heated flavour and odour of the milk change due to loss of dissolved gases like CO_2, O_2, resulting in cooked flavour.

7. The foul smell and rancid taint developed by dairy products is due to liberation of free fatty acids by hydrolysis or due to enzymes. This process is retarded by butyric acid but contributes to the foul smell, due to action of the enzyme lipase.

8. All solids in milk are referred to as total solids, and solids without fat as milk solids non-fat (MSNF).

9. Milk develops acidity, due to fermentation of lactose to lactic acid by streptococcus lactis. This is known as souring of milk. The taste changes from sweet to sour. At an acidity of 0.5 to 0.7%, curdling occurs. The lactic acid formed reacts with calcium of casein, to form ca-lactate which is soluble, and a precipitate of free casein is formed. Natural acidity is less than developed acidity, CO_2 acidity is 0.01-0.02%, citrate acidity is upto 0.01%, casein 0.05-0.08%, albumin <0.01% and the remaining is due to phosphate which with casein forms the main titratable acidity.

1.9 NUTRITIONAL VALUE OF MILK

Milk is nearly a complete food except that it is some what deficient in iron, copper and vitamin C. It has very high nutritive value and gives about 75 to 100 kilo calories per 100 gm.

(1) Milk has sufficient quantity of major proteins like casein, α-lactalbumin, β-lactoglobulin and bovine serum albumin. They contain all essential amino acids and are useful in body building and other normal cell functions.

(2) The major milk sugar is lactose, whose primary function is to supply energy, but it also helps to establish mild acidic reaction in the intestine which check growth of unwanted bacteria in the intestine and facilitates assimilation of minerals.

(3) Milk fat has high energy value of 9.3 kilo calories/gram. Besides, it contains significant amounts of essential fatty acids. Apart from this, fat contributes to the flavour and physical properties of milk which increase consumer's preference for the milk.

(4) Milk is an excellent source of essential minerals especially calcium and phosphorus, which are very useful for bone formation. On an average milk contains—Ca-123, Mg-12, PO_4-95, Na-58, K-141, Cl-119, S-30, and citrate acid-160 mg/100 ml respectively. Milk is low in iron, copper and iodine. These minerals also play very important role in many physiological functions.

(5) Milk is a good source of vitamin A, D, thiamine and riboflavin. However, it is deficient in vitamin C.

(6) Milk contains several enzymes like lipase, esterase, phosphatase, proteases, amylase, xanthine oxidase, lactoperoxidase, catalase, aldolase, ribonuclease etc.

(7) Milk also contains non-protein nitrogenous substances, some flavouring substances, phospholipids, sterols, pigments etc.

1.10 NATURAL ANTIMICROBIAL SYSTEMS IN MILK

Several natural antimicrobial systems are detectable in milk operating either for the protection of the mammary gland from infection, or conferment of disease resistance on the suckling young. These natural inhibitory systems can also help preserve milk for some time, in natural form. One of the antimicrobial systems—LP system has been investigated thoroughly for extending the shelf-life of raw milk. Both specific and non-specific types of substances are secreted in milk as discussed below—

1.10.1 Immunoglobulins

These are the types of antibodies for pathogenic bacteria, which are produced locally in the udder (IgA) or transferred to milk from circulation (IgG). These immunoglobulins help in neutralising toxins, inhibiting bacterial adhesion, suppressing bacterial growth and aiding in the process of phagocytosis.

1.10.2 Leucocytes

Total cell count of milk from uninfected udder ranges from 1 to 5 lakhs/ml, of which 10% are polymorphonuclear leucocytes (PMN). These cells inhibit invading bacteria by phagocytosis.

1.10.3 Complement

About nine components of complements are found to be present in human milk and they are associated with bactericidal properties of milk.

1.10.4 Bifidus factor

Bifidus factor is a nitrogen containing oligosaccharide present in human milk, which supports growth of Bifidobacteria in infants. This organism helps in maintenance of intestinal health.

1.10.5 Lactoferrin

Lactoferrins are iron binding proteins, which inhibit the growth of bacteria by depriving them of iron. The concentrations of lactoferrin in human and bovine milk are 2-4 and 0.02-0.35 mg/ml, respectively.

1.10.6 Lysozyme

Most Gram positive bacteria are sensitive to lytic action of lysozyme. It is present in much higher concentration ($\equiv$ 30 mg/100 ml) in human milk than bovine milk (0.01 mg/100 sml).

1.10.7 Lactoperoxidase/Thiocyanate/Hydrogen Peroxide System

Lactoperoxidase is an enzyme naturally secreted in milk in concentration of about 30 μg/ml. Thiocyanate is the substrate for this enzyme to act and is present in varying concentrations at 1 to 10 ppm depending on the feeding pattern of the animal. The third component of the system is hydrogen peroxide which acts as a catalyst. Under normal conditions it may be supplied by hydrogen peroxide producing organisms within udder (e.g. Streptococci) or by the polymorphoneutrophils. When all the three components are available, the system gets activated and produces unstable substances which are bacteriostatic.

$$SCN^- + H_2O_2 \xrightarrow{\text{Lactoperoxidase}} HOSCN + OH^-$$
$$\text{(Hypothiocyanous acid)}$$
$$\downarrow \text{Dissociate}$$
$$OSCL^-$$
$$\text{(Hypothiocyanate ions)}$$
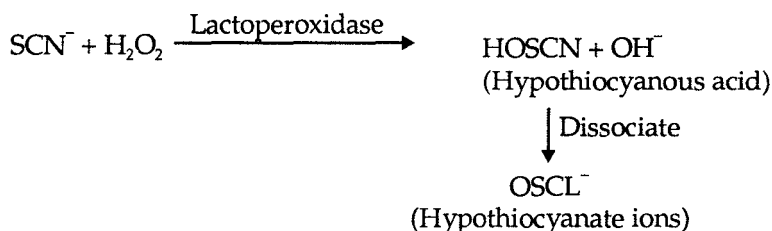
The hypothiocyanate ions adversely affect cell membrane causing inactivation of several vital metabolic enzymes and leakage of potassium ions and amino acids. When excess hydrogen peroxide is available, the reaction proceeds further as under—

$$HOSCN + H_2O_2 \cdots\cdots\blacktriangleright HO_2SCN + H_2O$$
$$\text{(Cyanosulphurous acid)}$$

$$HO_2SCN + H_2O_2 \cdots\cdots\blacktriangleright HO_3SCN + H_2O$$
$$\text{(Cynosulphuric acid)}$$
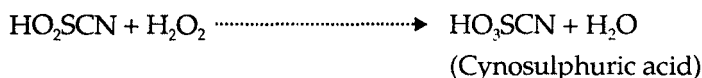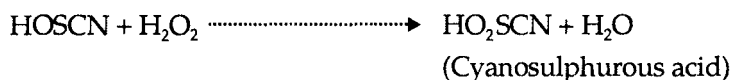
The ionic forms of above compounds possess baetericidal activity.

The inhibitory effect of this system is both species and strain specific. Against some Gram negative bacteria and group A Streptococci, the effect is bactericidal, while against other Gram positive organisms, the system is bacteriostatic.

The end products of the final reaction are water, ammonia, sulphate and carbon dioxide, which are stable.

Attempts are being made to use this system for preservation of raw milk, especially in tropical countries like India, where immediate cooling facilities are not available. However, hydrogen peroxide and thiocyanate are deficient in milk and hence are required to be added externally. This needs legal clearance and hence it is necessary to educate the users of the system and get the legal act modified permitting the addition of only a few ppm of these two components. This system is not recommended as a substitute of pasteurization, but as a means to prevent souring and deterioration before it is brought to the processing dairy plants. Extension in shelf-life upto 8-13 h at room temperatures have been observed with varying concentration of thiocyante and hydrogen peroxide (Table 1.6). The effect of LP-system and sanitization of milk cans on bacteriological counts and keeping quality of milk can be seen from Table 1.7.

**Table 1.6 : Keeping quality of raw milk (as determined
by C.O.B. test) treated with LP system**

Treatment	Keeping quality in hours at		
	37°C	30°C	23°C
Control	3.9	5.5	7.5
SCN:H$_2$O$_2$ in			
10 : 10 ppm	7.4	9.9	14.2
20 : 20 ppm	8.9	12.2	16.2
30 : 30 ppm	10.3	13.6	19.2

Source : Thakar, R. P. and Dave, J. M. (1986). Milchwissenschaft 41(1): 20-22.

**Table 1.7 : Effect of sanitizing milk can and
LP-treatment on quality of raw buffalo milk**

	Milk in Unsanitized cans		Milk in Sanitized cans	
	Control	LP* treated	Control	LP* treated
Standard plate count/ml ($\times$ 10^6)	14	5.0	0.45	0.083
Keeping quality of milk at 37°C in hours	5.5	8.5	6.5	9.5

* Treated with 15 ppm sodium thiocyanate and 10 ppm of hydrogen peroxide, 3 h after milking.

Source   : Chakraborty, B. K., Chaudhry. S. S., Alex. K. A., Jacob, G. and Soni, G.J. (1986). Milchwissenschaft 41(1): 16-19.

1.11 SCOPES FOR NEW DEVELOPMENTS IN DAIRY INDUSTRY

There are brighter horizons which must be explored for product diversification because dairy organisations making traditional fermented products are facing severe onslaught of market saturation within the country and dumping of stocks by the advanced countries of the world.

The manufacture of fermented milks, nowadays is mainly based upon traditional technologies. All these products are made by adding the appropriate starter cultures to milk and allowing them to grow until the milk is fermented to the required degree. In addition to this basic technology dairy industry takes appropriate measures to control the manufacture and improve the quality of fermented milks. However, new trends are becoming apparent with regard to consumer acceptance of fermented milks and these trends are accelerating at a faster rate. The conclusions of the 22nd International Dairy Congress (1988) with regard to fermented milks emphasized some of the emerging trends as mentioned below.

- Nowadays, a large variety of fermented milks is produced because of their good organoleptic properties; in near future health properties will become more relevant.

- At present, microorganisms for the production of fermented milks are selected for their growth characteristics in milk. However, in near future selection will also be based upon interaction with gastrointestinal microflora and metabolic properties.

- The influence on the immune system and the depression of enzymes involved in the development of cancers are new areas of scientific interest which need further elucidation.

- In the future, genetic engineering of microorganisms and new technological possibilities will allow the creation of new fermented milks in which the desired properties are emphasized.

- New products should be developed while the traditional ones should be maintained and improved.

  These trends have been defined for dairy microbiologists and technologists and the question that can be posed is how can the dairy industry fulfil these demands. In this context, introduction of new technologies assumes added significance. This means that new technologies have to be developed to make special fermented products. There are several ways to realise these targets. The main methods by which it can be accomplished are—

- The use of membrane techniques.

- Separate cultivation of starter organisms followed by special handling and mixing.

- Production of partly fermented milks.

Other potential areas include special fermentation products like the milk foods with probiotic approach and with immuno-modulating activity which will receive greater consumer attention as the market demand for value added fermented products is expected to be very high in the near future. Similarly, *Bifidobacterium* derived fermented products have proven excellent physiological activity in the infant digestion metabolism and nutrient utilization which exactly mimic breast feeding. Conditions can be further optimized for the enzymatic hydrolysis of lactose using β-galactosidase in concentrated food formulations for lactose intolerant infants as per the specifications and nutritional standards of WHO.

Milk based formulae with bioactive peptides of immunological significance can be prepared by fermentation process to mimic the biological attributes of breast milk. Dairy industry involved in the manufacture of fermented milks is expected to take a big leap in 21st century to produce high quality fermented foods catering to the needs of the consumers as a result of advancements in automation and computer based software programming, for controlling the various processing steps.

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MICROBIOLOGY OF RAW MILK

2.1 INTRODUCTION

Milk is considered to be the 'most nearly perfect' food for man and hence is one of the most important articles of the diet. It is an extremely complex mixture and usually contains about 87% liquid (water) and about 13% solid. Of the solids, there is an average of about 4% fat and 9% solids other than fat. The latter consists of protein (about 3.3%), lactose (about 5%), and the ash content about 0.7%. The protein includes casein and albumen. There is usually about four times as much casein as all the other protein constituents of milk taken together. In addition to these, there are hydrolytic enzymes too. Milk serves as an excellent medium for the growth of many bacteria and is contaminated from various sources. Microorganisms play an important role in dairy industry. The quality and conditions of production of milk can be judged by microbial contents. If microorganisms are allowed to grow and multiply in milk, they produce chemical changes that make it unpalatable. Pathogenic bacteria can grow very well in milk, therefore, milk, may serve as a medium for the dissemination of infectious diseases. Besides, there are certain microorganisms which bring about chemical changes useful in the manufacture of dairy products.

In this chapter, microbiology of raw milk is discussed under sub-sections like sources of microorganisms in milk, Microflora of raw milk, Types of microorganisms occurring in milk, Raw milk testing and BIS grades of milk, etc.

2.2 SOURCES OF MICROORGANISMS IN MILK

There are several principal sources of contamination of milk. From the time the milk leaves the udder, until it is dispensed into containers, everything with which it comes into contact is a potential source of more microorganisms. Milking performed under hygienic conditions, with strict attention to sanitary practices, will reduce the entry of microorganisms into the milk. Naturally the fewer the organisms that can get into the milk, the fewer have

a chance to grow. In the following paragraphs the sources of microorganism in milk and the precautions to minimize their entry into the milk are discussed.

2.2.1 The Dairy Cattle—Milk Producing Animal

Unless the producing animal is clean, and her flanks, udder and teats given special sanitary care just before milking, her body can be source of considerable contamination. The first few streams of milk from each teat should be collected, separated and discarded. This flushes out the organisms that have entered the teat through the teat opening. Milk from a cow with an infected udder is likely to contain a large number of organisms. The probability of discases of the udder contaminating the milk is very high. *Mastitis,* which is a disease causing inflammation of the udder, contributes considerable number of organisms, sometimes even blood cells, into the milk. If all the milk is not drawn out of the udder but remains between milkings, it may spoil within the udder itself and thus increase the count considerably.

The primary causative organism, *Streptococcus agalactiae* is commonly present, even though no clinical evidence of mastitis is present in the cows. This organism is normal habitant of udder and is not pathogenic for humans. The other common mastitis causing organisms, potentially harmful are, *Strephylococcus aureus* and *Escherichia coli.* Occasionally, *S. uberis, S. dysagalactiae, Corynebacterium pyogenes* and some others are also found in mastitic infections.

The other pathogenic organisms which can come through animals include—*Mycobacterium tuberculosis, Coxiella burnetii, Brucella abortus* etc.

Washing and massaging the cow's udder with a warm detergent--sanitizer solution before milking serves to clean the area. The hair of all animals harbours organisms. The hair, dirt and dust often fall from the body into the milking pails or the teat cups of milking machines. The modern practice is to keep the flanks clipped to minimize contamination.

2.2.2 The Milking Area

The microbial content of air is greatly affected by many conditions and practices. Dried dirt and filth is picked up by air movements and carried about as dust in the atmosphere. For this reason, dust may be the source of almost every kind of contamination. The sprays which are sometimes used to cut down air contamination are not very useful. However, the main point of keeping the conditions clean and sanitary is not to raise dust.

Micrococci, Coryneform and Bacillus spores are likely to contaminate through this source. However, it is shown that air derived bacterial count does not exceed 5 cfu/ml of milk.

2.2.3 Utensils and Equipment

Utensils and equipments are known to be the greatest sources of contamination. They may account for as much as 100,000 to a billion organisms per millilitre. Pails, strainers, cans, pipes, bottles, and other equipment used for the handling of milk are sometimes not properly washed and sanitized. Organisms survive in the cracks, corners, crevices, dents, scratches, and other irregularities of the utensils. Such neglect affords ideal conditions for the growth of microorganisms before the utensils are used again. Suitable washing procedures are facilitated by using warm water, a brush, and a detergent satisfactory to the

hardness of the local water used for cleaning. Subsequent sanitizing treatments may utilize hot air, hot water steam, chlorine or quaternary ammonium compounds.

When mastitis is prevalent in the herd, the utensils may contain *Str. agalactiae, Staph. aureus, E. coli* or *Corynebacteria*. When hot cleaning of equipments is done, the predominating organisms are thermoduric, mainly *Bacillus spp., Microbacterium spp.* and *Micrococcus*. When cold cleaning is followed, a heterogenous microflora are present.

2.2.4 Milking Machine

Milking machines are used when large number of animals are to be milked at a time. As milk production in India is on very small scale per producer, the milking machines are rarely used. However, when they are used, proper care is required not only in its cleaning but also in its use, because its improper use may damage the udder and thereby increase the risk of contamination.

In a survey in Wales (U.S.A.) about 20% of milking machines clusters had shown rinse count of $> 1 \times 10^9$ cfu/cluster. This shows its significance as a source of contamination. When pipe lines reused for direct milk collection, their improper cleaning and sanitation add further to the microbial load of milk. The types of microorganisms coming from milking machines are similar to those coming from other utensils and equipments.

2.2.5 Milker Personnels

All persons involved in the milking process must be in good health and must be careful in their personal cleanliness. They should wash their hands, clean and rinse them with an effective bactericidal solution, and dry them with a clean towel before starting, and frequently during work, and immediately following every rest stop. They should keep finger nails free of dirt. Each person must always carry a clean handkerchief and use it to prevent the spraying of nasal and oral discharges into the atmosphere, equipment , or products. They should wear a neat and clean uniform. A surgical mask is an effective addition to the uniform. Although workers may not contribute a large number of organisms, these are of considerable importance since they may well be human pathogens. Probably the majority of milk borne epidemics of disease were started by workers who were carriers, or who had mild cases, or who were in close contact with others so affected. The importance of contamination contributed by the workers is simply that it is the most dangerous.

The possibility of diseased milkers contributing to pathogenic organisms is of great importance. *Streptococcus* spp. and *Corynebacterium diphtheriae* may be transmitted directly from the throat to the milk by coughing or sneezing. Dysentery bacilli may contaminate the milk through hands of the person concerned.

2.2.6 Water Supply

Water is continuously required for cleaning and processing operations in a dairy. Special care or treatment must be taken to supply good quality water. Water quality will vary with the source of supply. Water from surface supplies is contaminated by dust, animals, plants, people, and other agents. The microbiological quality of water is tested before it is used in a dairy plant. The most common test for water destined for domestic or industrial use, is the *presumptive coliform test.* This test reveals *faecal* or *sewage* contamination. Chlorination of water is commonly practised to assure potability.

If water is not properly treated, the water may add coliforms, faecal streptococci, *Clostridium* spp., *Pseudomonas* spp. and less frequently coryneforms, *Bacillus* spores and lactic acid bacteria.

Keeping in mind the sources of contamination the following measures are suggested for hygienic milk production.

1. **Health care of animal**—Vaccination and regular check for disease are essential. Infected animal should be separated from herd and its milk should not be mixed with the bulk.

2. **Proper cleaning of animal**—Periodic clipping plus daily brushing and at least washing of udders and teats of the animal are advisable practices to keep the animal clean.

3. **Maintaining a sanitary barn**—it should he properly constructed, kept clean, adequately ventilated and kept free from flies and insects. Accumulation of dirt, dust, manure, urine should not be allowed. Avoid using dusty bedding and do not brush the animal just before milking. Don't sweep a short time before milking and avoid handling hay and feeds just prior to milking.

4. **The milker** should be healthy, free from contagious disease and should have a sense of cleanliness and personal hygiene. The hands should be washed and dried with clean towel before milking.

5. **The milking utensils** should be preferably made of stainless steel or aluminium alloy with smooth surface, free from dents, pittings and can be cleaned easily. The equipment cleaning should be done as follows:
   - Rinse the equipment immediately after use.
   - Clean it with detergent.
   - Rinse again for removal of detergent.
   - Just before use, sanitize by hot water or chemical sanitizer. The empty utensils should be kept inverted and dry.

6. Assure the use of **high quality potable water** or use it after treating with chemical sanitizer like chlorine.

2.3 MICROFLORA OF RAW MILK

The numbers and types of microflora in raw milk depends upon several factors, starting from the health of the animal to conditions of production, handling, storage and transportation. When the milk is cooled immediately after production and stored below 5°C, it prevents bacterial multiplication and reflects the microflora, similar to that present initially. The total count in raw milk may vary from less than 1000 to more than 10,00,000 per ml. This is routinely estimated by standard plate count (SPC) by plating milk on tryptone dextrose agar and incubating at 37°C for 48 h. Table 2.1 shows an early survey of bacteriological quality of raw milk in India, produced under different conditions. The season also has great influence on the number of microflora in milk. As per the guidelines of International Dairy Federation (IDF), the production of milk having SPC consistently less than 10,000 per ml reflects good hygienic practices while high initial SPCs of more than 100,000 per ml are evidence of serious faults in production hygiene. Table 2.2 shows the count of different groups of microorganisms in milk collected from various sources in Gujarat.

Table 2.1 : Bacteriological quality of raw milk produced under different conditions in India

Source of sample	Age of milk from time of production (h)	Standard Plate count (X10^6/ml)					
		Centre A			Centre B		
		Winter (15-25°C)	Summer (22-35°C)	Monsoon (20-32°C)	Winter (9-25°C)	Summer (28-45°C)	Monsoon (23-45°C)
Organized farm (bulk milk)	2	0.10	0.17	0.18	0.40	0.91	0.9
Village Produced	2	0.34	0.58	0.47	0.74	2.62	1.1
Private Dairies	4-5	3.41	3.68	1.47	0.19	9.80	1.3
Milk vendors in town	4-5	1.59	1.99	3.75	1.21	6.66	2.4

Average data for 200-300 samples in each category

Source   : Laxminarayan, H.; Iyengar, M.K.K. and Iya., K. K. (1959). XI Int. Dairy Congress. 1 : 374

Table 2.2 : Microflora of raw milk in Gujarat

Types	Individual Producer		Collection Centre		Dairy Plant (Pooled)
	Cow	Buffalo	Cow	Buffalo	
Total Plate count (×10^6 per ml)	7.9	32	9.1	5.1	17
Coliform Count (×10^4 per ml)	0.63	2.8	66	22	150
Acid producers count (×10^4 per ml)	30	9.6	13	5.9	290
Mesophilic Aerobic spore Count (per ml)	208	328	183	393	493
Sulphite reducing clostridial count (per ml)	0.74	0.74	0.76	0.49	2.5

Source : Siva, C. V. (1991). M. Sc. Thesis, Guj. Agril. Univ., Anand.

The microflora of milk can be grouped as normal and abnormal flora. Normal flora can be considered as those which come naturally from udder. These are mainly Micrococci, Streptococci and Corynebacteria. Abnormal flora are those, which are getting entry from various sources. However, better way of classifying them is based on their biochemical characteristics and temperature relationships. Grouping based on temperature relationship of microflora is most practical because the relative dominance of a particular group will depend on the storage temperature and processing conditions of the milk.

Table 2.3 shows the relative proportions of different types of microflora in raw milk. Based on temperature relationship, the types of microorganisms in milk can be grouped as psychrotrophic, mesophilic, thermoduric and thermophilic.

Table 2.3 : Incidence of the main groups of microorganisms in low count raw milk

Sr. No.	Group	Types Involved	% incidence
1.	Micrococci	*Micrococcus*	30-99
		Staphylococcus	
2.	Streptococci	Enterococcus	0-50
		Group N and	
		Mastitis Streptococci	
3.	Gram positive asporogenous rods	*Microbacterium*	< 10
		Corynebacterium	
		Arthrobacter	
		Kurthia	
4.	Gram negative rods	*Pseudomonas*	< 10
		Acinetobacter	
		Flavobacterium	
		Enterobacter	
		Klebsiella	
		Aerobacter	
		Escherichia	
		Serratia	
		Alcaligenes	
5.	Gram positive spore formers	*Bacillus*	< 10
		Clostridia	
6.	Miscellaneous	*Streplomyces*	< 10
		Yeasts	
		Molds	

2.4 TYPES OF MICROORGANISMS OCCURRING IN MILK

As discussed earlier, there are various sources through which the microorganisms contaminate the milk. The magnitude and diversity of the microbial population varies considerably and is dependent upon the specific conditions associated with the production of a particular milk. The microorganisms occurring in the milk can be grouped into three main categories on the basis of their important characteristics—

- Biochemical types
- Temperature characteristic types
- Pathogenic types

2.4.1 Biochemical Types

This group consists of those microorganisms occurring in milk which bring about biochemical changes in it. They are—
- Acid forming microbes
- Gas forming microbes
- Ropy-milk forming microbes
- Proteolytic microbes
- Lipolytic microbes

(A) Acid Forming Microbes—Acid forming microorganisms are certain bacteria which bring about natural fermentation of milk. The most common type is the lactic acid fermentation which takes place during souring of milk under natural conditions. Milk of good sanitary quality when kept under conditions that allow growth of *Streptococcus* spp. *(e.g., S cremoris)* and *Lactobacillus* species *(e.g., L. casei, L. plantarum, L. brevis,* and *L. fementum)* develops a clean, sour flavour. *Streptococcus* spp. ferment lactose quickly but do not produce as high a concentration of lactic acid as members of the- genus *Lactobacillus*. *Micrococcus* species, *e.g., M. luteus, M. variens* and *M. freudenreichii* produce small amount of acid from lactose fermentation and sour the milk. *Escherichia coli* and *Enterobacter aerogenes* also ferment lactose to a mixture of end products like acids, gases and some neutral compounds. These are considered undesirable as they produce CO_2, H_2 and unpleasant flavour. *Microbacterium lacticum* is also reported in milk and ferments lactose to lactic acid and other end products.

(B) Gas Forming Microorganisms—There are certain bacteria like *Clostridium butyricum* which ferment lactose to acids accompanied with accumulation of gases, the gas being usually a mixture of CO_2 and H_2. *Clostridium butyricum* produces large amount of CO_2 whereas coliform bacteria produce H_2 in addition. Certain yeasts, *e.g., Torula cremoris, Candida pseudotropicalis,* and *Torulopsis sphaerica* are reported in milk. They, too, ferment lactose and produce CO_2.

(C) Ropy-milk Forming Bacteria—The conversion of liquid milk to viscous material by the action of microbes is called 'ropy fermentation'. These microorganisms synthesize a viscous polysaccharide material that forms a slime layer or capsule around their cells. *Alcaligenes viscolactis, Enterobacter aerogenes, Streptococcus cremoris,* and some species of *Micrococcus* are responsible for ropy fermentation. Ropy milk is not deleterious to health but is usually objectionable due to its appearance and is frequently used as the culture medium.

(D) Proteolytic Bacteria—*Bacillus subtilis, B. cereus* var. *mycoides, Pseudomonas putrefaciens, P. viscosa, Streptococcus liquefaciens,* and *Proteus* spp. are the proteolytic bacteria present in the milk. These microorganisms hydrolyse milk protein and increase the pH. Proteolysis may be preceded by coagulation of the casein by the enzyme rennin elaborated by bacteria resulting in the formation of soluble form of casein. Proteolysis degrades the casein to peptides which may be further degraded to amino acids which are responsible for alkaline reaction and bitter taste of milk.

(E) Lipolytic Microorganisms—Some of the microorganisms produce enzyme (lipases) which split milk fat to glycerol and fatty acids. Some of these fatty acids have a sharp flavour which causes imparting rancid flavour and odour to milk. Lipolytic microorganisms present in the milk are the bacteria *Pseudomonas fluorescens, Achromobacter lipolyticum;* yeasts, *e.g., Candida lipolytica;* and moulds, *e.g., Pencillium* spp. and *Geotrichum candidum.*

2.4.2 Temperature Characteristic Types

On the basis of their optimum temperature for growth and heat resistance, the bacteria encountered in milk are of the following four types—

(A) Psychrotrophic Microflora—In dairy industry, psychrotrophs are defined as those organisms which are able to grow below 7°C, regardless of their optimum temperature. This group is enumerated on standard plate count agar at 5-7°C in 7-10 days. Usually this group has an optimum temperature around 20°C. This group includes, both, Gram positive and Gram negative rods. Gram negative rods comprise about 10-15% of the initial total flora and include mainly *Pseudomonas, Acinetobacter, Flavobacterium. Achromobacter, Alcaligenes* and *Enterobacter*. About half of these flora is contributed by only *Pseudomonas*. They are especially significant in milk, because they are able to produce heat stable proteases and lipases in milk. Even though the cells are killed, their enzymes are not inactivated by pasteurization and their activity in milk leads to fat and protein degradation causing spoilage. The Gram negative rods mainly enter in milk from improperly cleaned utensils. They may also be derived from teat surface or water.

Certain species of Gram positive rods are also found to be present in raw milk, which belong to *Bacillus* sp., e.g. *B. coagulans, B. circulans, B. subtilis* and *B. megaterium*. The spores of these organisms and their enzymes are heat resistant.

(B) Mesophilic Microflora—The microorganisms having their optimum temperature between 20 to 40°C are generally regarded as mesophilic. These organisms are enumerated by plating milk at 30-38°C. The major groups include *Micrococci, Staphylococci, Streptococci, Microbacterium, Corynebacterium, Lactobacillus, Bacillus, Coliforms* and *Pseudomonas*. They come in milk from various sources, rapidly grow and spoil the milk when it is stored at room temperature.

(C) Thermoduric Microflora—Thermoduric microorganisms are defined as those, which are able to survive pasteurization treatment but not able to grow at pasteurization temperatures. The count of this group is determined by plating the milk after laboratory pasteurization (63°C for 30 mm.). Among the major groups present in milk, *Microbacterium* shows 100% resistance to pasteurization. The other abundant group is Micrococci, but show less than 100% resistance. Other groups are *Bacillus, Clostridia, faecal Streptococci*, etc.

These organisms come from animal, utensils or environment and constitute a major flora of pasteurized milk.

(D) Thermophilic Microflora—The organisms which survive and grow at pasteurization temperature are regarded as thermophilic. They have optimum growth temperature around 55°C and grow upto 70°C. Therefore the enumeration of these organisms is done by incubation at 55°C. Very few organisms of this type may enter in milk from soil, bedding or feeds. The members of this group in raw milk belong to spore forming Gram positive rods e.g. *Bacillus stearothermophilus, B. circulans* and *B. thermoacidurans*. If proper cleaning of pasteurizer is not done, this group can grow there and contaminate incoming fresh milk.

2.4.3 Pathogenic Types

A variety of diseases are potentially transmissible through milk. The source of disease causing microorganisms occurring in milk may be either the dairy cattle or humans.

Diseases transmitted through milk either from infected cows or other sources are listed below—

Source	Diseases		Causal organism
	Cow	Man	
Cow	Tuberculosis	Tuberculosis	*Mycobacterium tuberculosis*
	Mastitis	Sore throat	*Micrococcus piogenes*
	Brucellosis	Undulant fever	*Brucella abortus*
	Anthrax	Anthrax	*Bacillus anthracis*
Other	—	Typhoid	*Salmonella typhi*
		Diphtheria	*Corynebacterium diphtheriae*
		Scarlet fever	*Streptococcus pyogenes*
		Q. fever	*Coxiella burnetii*
		Cholera	*Vibrio coma*
		Enteric fever	*Salmonella paratyphi,*
			S. typhimurium

2.5 Raw Milk Testing and BIS Grades of Milk

The quality of raw milk is tested before acceptance at the dairy plant. These tests check the suitability of milk for further processing and human consumption. The milk is also graded on the basis of certain tests. In some developed countries, the system of certifying the herd is prevalent that enforces strict hygienic and animal health standards and certifies various grades of milk. However, in our country, the raw milk contains excessive number of bacteria because of the reasons stated earlier and a system of elaborate grading is not being followed.

The various routine tests employed for raw milk testing are given below—

A. Platform tests

    1. Smell and organoleptic test

    2. Clot on boiling test (COB)

    3. pH

    4. Titratable acidity (as % lactic acid)

    5. Sediment test

    6. Alizarin alcohol test

    7. 10 mm. resazurin test

    8. Direct microscopic count (DMC)

    9. Direct microscopic somatic cell count (DMSCC)

    10. Fat content

B. Microbiological quality tests

    1. Methylene blue reduction test (MBRT)

    2. Resazurin reduction test (RRT)

      3.   Total plate count (Standard plate count)

      4.   Coliform count

      5.   Thermoduric count

      6.   Thermophilic count

      7.   Psychrotrophic count

      8.   Spore count

      9.   Proteolytic count

      10.   Lipolytic count

  C. Others

      1.   Test for mastitis

      2.   Test for antibiotic residues.

Among all the above tests, smelling the milk in can at the receiving dock and COB, if needed, are routinely used to decide acceptability of the milk by the dairies in our country.

Clot-on-Boiling test is done by taking 2 ml milk in a test tube and boiling it over a burner flame. If precipitates or clotted particles appear, the milk is not accepted as it indicates developed acidity and such milk may clog pasteurizer plates.

Certain dairies also fix the standards for level of acidity, above which the milk may not be acceptable. Test is carried out by taking 10 ml milk in a porcelain dish and then adding 1 ml of phenolphthalein indicator: The contents are titrated against 0.1N NaOH, till light pink colour persists. The titratable acidity is generally expressed as per cent lactic acid and calculated as under—

$$\text{Titratable Acidity (\%)} = \frac{9 \times \text{mls of 0.1N NaOH used}}{\text{Volume of milk taken}}$$

Table 2.4 : Grades of raw milk as per BIS in India based on several tests

Grade	Direct Microscopic Count (DMC) per ml	Standard Plate Count (SPC) per ml	Methylene blue reduction time in hours	One hour Resazurin disc number	Presumptive coliform test (in 0.01 ml)
Very good	NS	<2 lakhs	>5	NS	Absent
Good	<5 lakhs	2 lakhs to 10 lakhs	3 and 4	4 or higher	Absent
Fair	5 to 40 lakhs	10 lakhs to 50 lakhs	1 and 2	3.5 to 1.0	Absent
Poor	40 to 200 lakhs	> 50 lakhs	<1/2	0.5 to 0	Present
Very poor	>200 lakhs	NS	NS	NS	NS

NS : Not specified

The microbiological quality is also routinely tested by MBRT or RRT, standard plate count and coliform count. However, these tests are not used still for pricing the milk in

India. The Bureau of Indian Standards (BIS) is the official organisation in India for voluntary standards and it has laid down standards for grading raw milk based on several tests (Table 2.4).

2.6 EFFECT OF COOLING, STORAGE AND TRANSPORTATION ON THE MICROFLORA OF MILK

The following four factors will affect the microflora of milk after production:
1. Numbers and types of microflora initially present.
2. Temperature of storage.
3. Duration of storage.
4. Natural inhibitory systems in milk.

Generally, animals are milked twice a day world-wide. In advanced dairy countries, it is a common practice to cool the milk immediately on the farm, and the same is collected by the dairy plants every day or alternate day or twice a week. This practice requires cold storage of milk at the collection centre for 2-3 days, before it is processed. Under these conditions, the psychrotrophic microflora dominate in milk. This group is well known to produce heat resistant proteolytic and lipolytic enzymes in milk, which can lead to development of taints and can adversely affect on quality and quantity of products made using such milk.

In India, the conditions are quite different. We have a tropical climate, have no adequate cooling facilities and have small scale scattered production. Under the popular "Anand Pattern" cooperative system, the individual milk producer supplies the milk within 1-3 h of production to a village level society. This is transported twice a day in cans within 3-5 h to the district level dairy plant under ambient conditions. Where, the dairy plant is far off, the milk from village society goes to a chilling centre, where it is cooled to below 5°C and then transported to the district level dairy plant.

During first few hours after milking the microflora in milk do not multiply or may result in some reduction in numbers. This is due to the natural inhibitory systems of milk. The effectiveness of these system persists for longer time at low temperatures. However, after sometime, the number of microflora starts increasing depending upon temperature.

While storage at ambient temperature, mainly Streptococci and Coliforms become predominant. These groups increase the acidity in milk. This is the reason why souring followed by curdling with off odours is a common problem in our milk supplies. This group can continue to increase in numbers upto about 1% lactic acid production, after which it decreases due to inhibitory effect of lactic acid. Alongwith Streptococci and Colilforms, other Gram negative rods, Micrococci and spore formers also continue to grow till the developed acidity becomes inhibitory to them.

Cooling retards the bacterial multiplication of all types, especially of mesophilic souring causing microflora. However, longer storage under refrigerated conditions develop large numbers of psychrotrophic microflora. During transportation in cans at atmospheric temperature, all bacteria multiply. Agitation effect during truck transport also affects the growth of microflora alongwith partial churning of fat. It also results in breaking of some bacterial clumps leading to apparent increase in plate counts.

2.7 ANAND PATTERN

ANAND Pattern is a world famous 4 tier system of dairy development in India. In foundation, there are a large number of small milk producers in villages, who are members of village level Cooperative dairy (Generally called Society). Such societies form a district level union, having a processing plant. The third tier is a state level cooperative milk marketing federation, which looks into aspects of marketing of milk and milk products, with the aim of giving better benefits to the producer and ensuring supply of quality products to the consumer at reasonable rates. The profits gained goes in giving social and monetary benefits to the producers. The fourth component added recently is a cluster of slate level milk marketing federations called as National Cooperative Dairy Federation of India (NCDFI).

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3

MILK-BORNE DISEASES

3.1 INTRODUCTION

Milk is an excellent culture medium for the growth of a great variety of organisms. Pathogenic as well as saprophytic forms may remain viable and even multiply in milk. For this reason it is difficult to obtain pure milk and to keep it pure. The prevention of milk-borne disease is one of the most important problems of public health.

Pathogenic organisms of both bovine and human origin have been isolated from milk. Many serious epidemics were caused by the consumption of such products before this fact was clearly recognized. This is to be expected when one takes into consideration the enormous quantities of milk and its products that are consumed daily. Even today, epidemics are spread through milk, but they are of rare occurrence compared to the number reported during the early years of public health.

The abnormal changes that occur in milk are usually easily detected by appearance, taste, and smell. However, the presence of disease organisms cannot be detected in that manner. Milk containing disease bacteria looks and tastes normal and gives no warning to the consumer.

The disease organisms present in milk may be derived from (1) diseased cows/ animals or (2) persons collecting and handling milk.

3.2 DISEASES OF BOVINE ORIGIN

The health of the cow is a very important consideration, because a number of diseases of cattle, including tuberculosis, brucellosis, Q fever, salmonellosis, staphylococci infection, streptococci infection, and foot-and-mouth disease, may be transmitted to man through the medium of milk. The organisms of most of these diseases may get into the milk either directly from the udder or indirectly through infected body discharges which may drop, splash, or be blown into the milk.

3.2.1 Tuberculosis

Tuberculosis of cattle is produced by the bovine tubercle bacillus *Mycobacterium bovis*. The disease has been largely eradicated in this country but is still common in some parts of the world. The organism is very similar in appearance to the human type, being usually shorter and plumper.

Children, especially those under five years of age, are highly susceptible to the organism and may become infected by drinking milk from tuberculous cows. If the udders of cows are infected, contamination of the milk cannot be avoided.

If cows are suffering from tuberculosis of the lungs, the sputum is swallowed, with the result that the organisms appear in the faeces. Since most milk may contain some excreta, it is likely to show the presence of such organisms. It is doubtful that the organism multiplies in milk, but it can live and may retain its virulence for a considerable time.

The great reduction in the incidence of bovine tuberculosis in man in recent years indicates that, the practice of good sanitation in animal husbandry, the testing of cattle and removal of the reactors from the herds, and the pasteurization of milk have been effective in the control of this disease. The reservoir of bovine tuberculosis still exists, however; hence, constant vigilance against this disease must be continued by industry and health agencies.

3.2.2 Johne's Disease

The etiological agent is a small acid-fast rod known as *M. paratuberculosis*. The organism has been isolated from the intestinal mucosa of cattle suffering from chronic diarrhea. It is probably identical with the organism isolated from a similar disease in sheep.

M. paratuberculosis is an obligate parasite. It may be cultivated on artificial culture media only in the presence of dead acid-fast bacilli.

3.2.3 Brucellosis

Another disease organism found in cow's milk is *Brucella abortus*, which produces contagious abortion in cows. The organism may produce the same disease in mares, sheep, rabbits, and guinea pigs. Other organisms producing similar results are *B. melitensis* from goats and *B. suis* from hogs. *B. melitensis* may also infect cows and hogs and be excreted in milk. *B. suis* produces abortion in swine and frequently attacks horses, dogs, cows, monkeys, and laboratory animals.

The three organisms are pathogenic for man, producing the disease known as undulant fever or brucellosis (after Bruce, who first isolated *B. melitensis* from the spleen of patients who had died of the disease on the island of Malta).

Brucellosis may be contracted by drinking raw milk or, less frequently, certified milk. Pasteurized milk should be safe, since the organism is destroyed in the heat process. Because of this fact, many public health authorities believe that all milk should be pasteurized before it reaches the consumer.

The incidence of brucellosis in man is increasing at the present time, and a greater effort is required to reduce the extent of infection in cattle and its transmission to man through milk.

3.2.4. Q fever

This is a newly recognized disease of cattle which may be transmitted to man through the consumption of milk. Natural infections occur among sheep, cattle, and goats. The etiological agent is the rickettsial organism *Coxiella burnetii.*

Within the last few years, Q fever has reached endemic proportions in man in some parts of this country. A vaccine has been developed which protects cattle and probably laboratory personnel from infection. Pasteurization of milk is the most practical safeguard against its transmission to man through milk.

3.2.5 Salmonellosis

Members of the genus *Salmonella,* produce a variety of infections in man and animals. In man the organisms may give rise to (1) enteric fever, (2) gastroenteritis, and (3) septicemia.

Some species or strains are natural pathogens for cattle and other domestic warm-blooded animals, being present in the intestinal contents. Outbreaks of salmonellosis have occurred from drinking contaminated milk.

3.3 DISEASES OF HUMAN ORIGIN

Some of the important diseases of human origin that have been disseminated by milk are (1) typhoid fever, (2) scarlet fever, (3) diphtheria, (4) septic sore throat, (5) infantile diarrhea, and (6) poliomyelitis. The organisms may be transferred to milk by contaminated hands of the workers; by droplets expelled during coughing, sneezing, and talking; by moistening the hands with saliva during wet milking; and in other ways.

3.3.1 Typhoid Fever

Many typhoid epidemics have resulted from drinking milk contaminated with the organism *Salmonella typhosa.* Further investigations revealed that in each outbreak usually only one dairy supplying the milk was responsible for the epidemic. The organisms were introduced into the milk by typhoid carriers or unrecognized cases of typhoid fever among the workers at the dairies. The isolation of such individuals resulted in the disappearance of new typhoid cases in the communities. Epidemics of typhoid fever have seen traced to a lesser extent to the consumption of ice cream, cheese, and butter. Because of the improved sanitary conditions of production and handling of milk and milk products in this country, such epidemics are a rare occurrence today.

According to the U.S. Public Health Service—

Investigations of milk-borne disease outbreaks have shown that body discharges of infected milk handlers are the most frequent source of contamination responsible for such outbreaks. The diseases so transmitted through milk include typhoid fever, dysentery, diphtheria, septic sore throat, scarlet fever, and tuberculosis.

The health officer or a physician authorized by him, shall examine and take a careful morbidity history of each person connected with a pasteurization plant, or about to be employed by one, whose work will bring him into contact with the processing, handling, storage, or transportation of milk, milk products, containers, or equipment. If such examination or history should suggest that such person may be a carrier of, or infected with, the organisms of typhoid or paratyphoid fever, or any other communicable disease likely to

be transmitted through milk, he shall secure appropriate specimens of body discharges and cause them to be examined in a laboratory approved by him or by the State health authorities for such examinations, and, if the results justify, such persons shall be barred from such employment.

3.3.2 Scarlet Fever and Septic Sore Throat

Both diseases are produced by *Streptococcus pyogenes*, a pus-producing organism. Epidemics have been caused by the consumption of milk containing this organism. The milk may become contaminated by handlers or by infected udders. Usually a milker suffering from scarlet fever or sore throat infects the udders with the organisms by contaminated hands. The organisms rapidly multiply in the udders. Abscesses form and the milk becomes heavily contaminated. Such milk may produce septic sore throat or scarlet fever in persons consuming the product.

3.3.3 Diphtheria

The causative agent is *Corynebacterium diphtheriae*. The cells are straight or slightly curved rods and frequently swollen at one or both ends. They are nonmotile and gram-positive.

Diphtheria is spread principally by direct contact with human sources through either active cases of the disease or carriers. Although epidemics of milk-borne diphtheria are comparatively rare, a few outbreaks have been traced to infected milk handlers.

Pathogens transmissible through raw milk are listed in Table : 3.1.

Table 3.1 : Pathogens transmittable through raw milk

Sl. No.	Pathogens	Source	Significance/Disease caused in humans
1.	*M. tuberculosis* *M. bovis*	Animal	Tuberculosis
2.	*S. agalactiae*	Animal	Non pathogenic
3.	*Staph. aureus*	Animal, Human handler	Food poisoning
4.	*E. coli*	Animal Human handler	Gastro-intestinal disorders
5.	*Brucella abortus* *Br. melitensis* *Br. suis*	Animal	Brucellosis, Fever
6.	*Listeria monocytogenes*	Animal	Listeriosis
7.	*Leptospira-icterohaemorrhagiae*	Animal	Leptospirosis
8.	*Bacillus cereus*	Animal	Food poisoning
9.	*Clostridium perfringens*	Animal	Food poisoning Gas gangrene

... contd.

Sl. No.	Pathogens	Source	Significance/Disease caused in humans
10.	*Pasteurella multocida*	Animal	Pasteurellosis
11.	*Actinomyces* spp.	Animal	Non pathogenic
12.	*Coxiella burnetii*	Animal	Q fever
13.	*Salmonella* spp.	Human handlers. Faecal contamination of milk	Salmonellosis, Typhoid
14.	*Shigella* spp.	Human handlers, Faecal contamination of milk	Shigellosis, Dysentery
15.	*Vibrio cholerae*	Human handlers, Faecal contamination of milk	Cholera
16.	*Streptococcus pyogenes*	Human handlers	Sore throats
17.	Enterovirus	Human handlers	Diarrhoea, Poliomyelitis,
18.	Hepatitis virus	Human handlers	Hepatitis (liver disorders)
19.	Tick borne encephalitis virus	Animal	Biphasic milk fever
20.	Foot & mouth disease virus	Animal	Infection on hands
21.	*Yersinia enterocolitica*	Animal	Yersiniosis, Gastroenteritis

3.4 DISEASES OF MILKING ANIMALS

3.4.1 Mastitis

Mastitis is one of the costliest diseases confronting the American dairyman. It is estimated that the disease occurs in about one cow out of every four.

Mastitis is an inflammatory and, generally, highly communicable disease of the bovine udder. An infection may be acute, having a short but severe course which may lead to the loss of one or more quarters of the udder, or even death; or the infection may be chronic, flaring up at intervals, resulting in the eventual loss of part or the entire udder.

A number of organisms are involved, the most important being *Staphylococcus aureus*, *Streptococcus agalactiae*, and *E. coli*. Other organisms which have been isolated include *Corynebacterium pyogenes*, *Mycoplasma agalactiae* and possibly other species, *Pseudomonas*

aeruginosa, Streptococcus dysgalactiae and *S. uberis.* The organism most commonly associated with the disease is *S. aureus.*

Mastitis is harmful not only to the cow but also to man, since milk from an infected animal can be a means of spreading such organisms to man. If the mastitis is severe, pus and blood may appear in the milk. Milk containing appreciable numbers of the organisms and blood cells must be regarded as unfit for human consumption.

All organisms associated with mastitis are killed by pasteurization.

3.4.2 Foot-and-mouth Disease

This is a highly contagious viral disease of domestic animals. The virus produces fever, digestive disturbances, and a vesicular eruption on the mucous membranes of the mouth, on the skin between the toes, and on the udder and teats of the cow. From the vesicles, the virus may gain entrance to saliva, urine, faeces, and milk. The virus also has a consistent affinity to all areas of bovine skin even though gross cutaneous lesions usually are found only in pedal area.

The infection may be transmitted by feeding and drinking troughs, stalls, cattle cars, etc. The virus produces a high death rate among cattle; in man the disease runs a mild course.

Vaccines are now available for increasing the immunity in susceptible animals. One such vaccine is prepared by infecting monolayers of bovine kidney cells growing in flasks.

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4

PASTEURIZATION AND STERILIZATION OF MILK

4.1 INTRODUCTION

The destruction of all organisms in milk is called sterilization. The high temperature required to achieve this result would impart a cooked flavour to the milk. Such milk is objectionable for two reasons: (1) The cooked flavour is not so pleasant as that of unheated milk, and (2) heating to such a high temperature might result in a decrease in the vitamin content. These objections are largely overcome by heating milk to temperatures lower than that required to sterilize completely but sufficiently high to destroy all disease organisms likely to be present.

The destruction of disease and most other organisms in milk without attempting complete sterilization is called pasteurization.

According to the U.S. Public Health Service (1967):

The terms "pasteurization," "pasteurized," and similar terms shall mean the process of heating every particle of milk or milk product to at least 145°F., and holding it continuously at or above this temperature for at least 30 min., or to at least 161°F., and holding it continuously at or above this temperature for at least 15 sec., in equipment which is properly operated and approved by the health authority: *Provided*, that milk products which have a higher milk fat content than milk and/or contain added sweeteners shall be heated to at least 150°F., and held continuously at or above this temperature for at least 30 min., or to at least 166°F., and held continuously at or above this temperature for at least 15 sec.:

Provided further, that nothing in this definition shall be construed as barring any other pasteurization process which has been recognized by the United States Public Health Service to be equally efficient and which is approved by the State health authority.

4.2 PUBLIC HEALTH SIGNIFICANCE

The public-health value of pasteurization is unanimously agreed upon by health officials. Long experience conclusively shows its value in the prevention of diseases which may be transmitted through milk. Pasteurization is the only practical, commercial measure which, if properly applied to all milk, will destroy all milk-borne disease organisms. Examination of cows and milk handlers, while desirable and of great value, can be done only at intervals and, therefore, it is possible for pathogenic bacteria to enter the milk for varying periods before the disease condition is discovered. Disease bacteria may also enter milk accidentally from other sources, such as flies and contaminated water and utensils. It has been demonstrated that the time-temperature combinations of 143°F for 30 min., and 161°F for 15 sec, if applied to every particle of milk, will devitalize all milk-borne pathogens. Compilations of outbreaks of milk-borne disease by the U.S. Public Health Service, over many years, indicate that the risk of contracting disease from raw milk is approximately 50 times as great as from milk labeled "pasteurized."

A note of caution is in order. Although pasteurization devitalizes the organisms, it does not destroy the toxins that may be formed in milk when certain staphylococci are present (as from udder infections), and when the milk is not properly refrigerated before pasteurization. Such toxins may cause severe illness.

Numerous studies and observations clearly prove that the food value of milk is not significantly impaired by pasteurization.

The pasteurization process reduces the bacterial count 99 to 100 percent, depending upon the number and kinds present.

4.3 PRINCIPLES UNDERLYING PASTEURIZATION OF MILK

Pasteurization is one of the universally adopted process for making the milk safe for human consumption and increase its keeping quality. The name pasteurization is coined after Louis Pasteur, who demonstrated that heating wine at 50-60°C killed spoilage microorganisms. However, pasteurization of milk was first attributed to Dr. Soxhet of Germany in 1886.

The term pasteurization as applied to market milk today, refers to the process of heating every particle of milk to at least 63°C for 30 min. or 72°C for 15 s (or any other equivalent time-temperature combination) followed by immediate cooling to less than 5°C.

4.3.1 Basis of Pasteurization

The main objective of pasteurization is to make milk safe for human consumption by destroying all asporogenous pathogens in milk. The process also destroys about 99% of the total microorganisms leading to increase in shelf-life of milk. Among the pathogens, *Mycobacterium tuberculosis* was considered to be most heat resistant pathogen likely to come in milk. It resists upto 138°F (58.9°C) for 30 min. Keeping safety margin, the pasteurization temperature was kept as 143°F (61.7°C) for 30 min. However, later it was found that *Coxiella burnetii* may survive this pasteurization and, hence since 1956, the pasteurization temperature time combinations were fixed as 145°F (62.8°C) for 30 min or 161°F (71.7°C) for 15 s. Just below this temperature time combination, the alkaline phosphatase enzyme of milk gets inactivated. Hence, the negative phosphatase test has become very useful index

for checking the efficiency of pasteurization. Recently, sonic outbreaks of a disease called listeriosis in Canada, USA and UK from consumption of milk and cheese have raised concern about the effectiveness of pasteurization for eliminating the causative organism, *Listeria monocytogenes*. It is indicated that, while the free cells of *L. monocytogenes* are destroyed by usual pasteurization, the clumps or the cells present inside white blood cells may escape pasteurization process. Attempts are going on to check the adequacy of existing international standards for pasteurization against this.

4.3. 2. Methods of Pasteurization

Various methods employing different temperature time combinations and different type of equipments are available. However, two most common methods employed by dairies are described below.

(A) Batch Pasteurization (LTLT Method)

This method is also called as LTLT (Low Temperature Long Time) process, where milk is heated in a tank or containers in batch for 30 min at 63°C (145°F). The milk is cooled in the same container to <10°C immediately. Usually circulating steam or hot water is used as a medium of heat transfer in jacketed vessels. The method requires longer time and is not suitable where large quantity of milk is received.

(B) Continuous Pasteurization (HTST Pasteurization)

In this method, High Temperature Short Time continuous method of pasteurization, milk is heated upto 72°C (161°F) for 15 seconds and cooled immediately below 5°C. The method is rapid and most suitable for commercial dairies. The process is economical and saves energy as the principle of regeneration and efficient heat transfer is used in the equipment design, which is composed of a number of thin plates.

A schematic diagram of HTST method of pasteurization is shown in Figure 4.1.
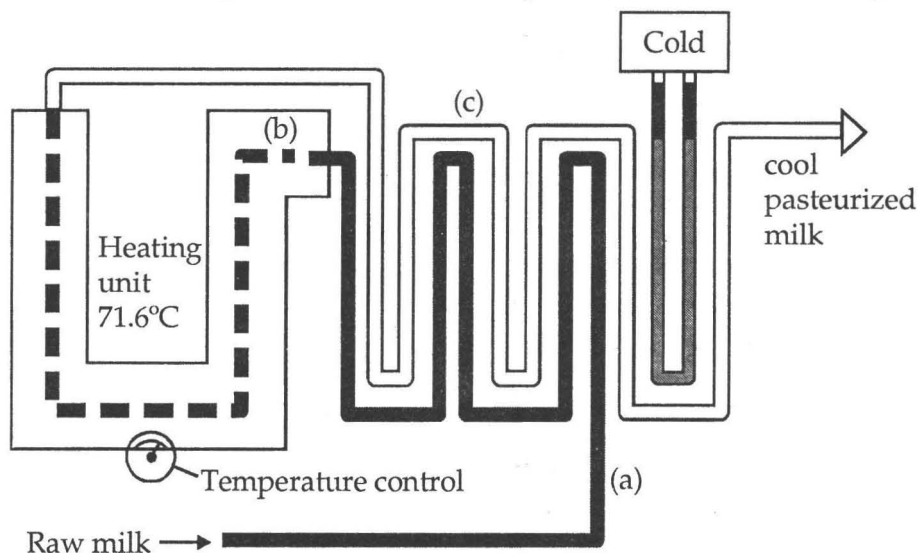
Fig. 4.1 : A schematic diagram of HTST method of Pasteurization.

4.3.3 Phosphatase Test

Phosphatase is a thermolabile enzyme present in raw milk and in many tissues, which is inactivated by adequate pasteurization process.

The phosphatase test is, therefore, employed to determine the efficiency of the pasteurization process. The test is based upon the property of the enzyme to liberate phenol from phosphoric-phenyl ester added to a sample of milk. The amount of phenol liberated is estimated by the addition of 2:6. dichloroquinonechlorimide (CQC), which turns blue in presence of phenol. The reaction is summarized in the following equation:

Disodiumphenyl phosphatase → phosphate + phenol + CQC (blue colour)
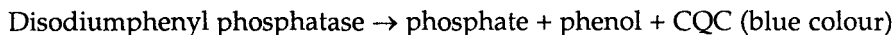
Phenol is converted to indophenol blue. The indophenol blue is extracted by shaking with neutral n-butyl alcohol and the concentration determined quantitatively by comparing with a series of colour standards in a colorimeter. When the milk is heated at 145°F or above for 30 minutes, all the enzyme present is destroyed. The presence of the enzyme indicates that the milk has been underheated, or heated for a short period, or there is subsequent addition of raw milk. Disease organisms likely to be present in milk are killed at a temperature lower than that required to inactivate the enzyme. Therefore, a heat treatment adequate to inactivate the enzyme should ensure a milk that is free from common pathogenic bacteria. Several harmless bacteria such as members of the genera *Aerobacter, Micrococcus* and *Klebsiella* produce thermostable phosphatase. This can give false positive results in properly pasteurized milk.

4.3.4 Microflora of Pasteurized Milk

The microorganisms present in pasteurized milk can be from two sources; (i) thermoduric organisms which have survived the heat treatment, (ii) contaminants coming after pasteurization, from inadequately cleaned milk contact surfaces of equipments or front containers in which the milk is packed. When adequate temperature-time combination during pasteurization process is not maintained, the other heat sensitive microorganisms from raw milk may also come in pasteurized milk. Subsequent to pasteurization, the numbers and the relative dominance of a particular type will largely depend on the temperature at which the milk is held.

Generally total count of 20,000/ml is achievable in pasteurized milk. However, it will vary largely based on initial raw milk quality and extent of contamination. Among the thermoduric microorganisms in milk, species of *Microbacterium, Arthrobacter, Streptococcus, Micrococcus* and *Bacillus* are main. *Lactobacillus* is not frequently encountered. As this group largely comes from improperly cleaned equipments, the thermoduric count in pasteurized milk is considered as an index of equipment sanitization on the producing farm.

Thermophilic organisms of *Bacillus* sp. become problematic in pasteurized milk when a part of milk is held for some time at 50-70°C. Repeated use of batch pasteurizer without cleaning in between may result in build-up of thermophiles.

Aerobic spore forming bacilli mainly *B. subtilis, B. cereus, B. coagulans, B. megaterium,* etc. are forming a major portion of psychrotrophic microflora in pasteurized milk. Other Gram negative psychrotrophic rods, like *Pseudomonas* and *Flavobacterium* come as post-pasteurization contaminants. This group becomes a major spoilage group in pasteurized milk stored at low temperature. Coliforms are destroyed by adequate pasteurization, hence

their presence in pasteurized milk indicates post-pasteurization contamination or faulty pasteurization.

4.3.5 Shelf-life and Spoilage of Pasteurized Milk

The shelf-life of milk is mostly determined by the off flavour development or instability resulting from bacterial growth which makes it unacceptable to consumer. Usually the end point of shelf-life will be when a count of 10^6-10^7 organisms/ml is reached, irrespective of the predominant organism. The factors which affect the shelf-life are:

(i)   Temperature of pasteurization,

(ii)   Types and number of each type of organisms present in milk after pasteurization,

(iii) Temperature of storage, and

(iv)   Packaging conditions.

The keeping quality of not more than 2 weeks have been observed in pasteurized milk at very low temperatures. Generally it is observed that increase of about 3°C in storage temperature, reduces the keeping quality by half. Indian scientists have observed shelf-life of 7.3, 28.3 and 57.1 h at storage temperatures of 37, 22 and 15° C. respectively.

Gram negative rods produce bitter flavour and unclean taints at a population of about 10^7 organisms/ml. *B. cereus* will produce bitterness at slightly low count i.e. 10^6/ml. When acid producing organisms like *Lactococcus lactis* or *Streptococcus thermophilus* grow, it leads to souring and coagulation. Protein destabilization also brings about viscosity changes in milk.

4.3.6 Microbiological Tests and BIS Standards for Pasteurized Milk

The pasteurized milk is routinely analysed for standard plate count, coliform count, MBRT and alkaline phosphatase test. Table 4.1 shows BIS requirements for the same.

Occasionally, the milk can also be analysed for thermoduric, thermophilic, psychrotrophic, proteolytic and lipolytic organisms.

Table 4.1 : BIS standards for pasteurized milk

Sl. No.	Test	Requirement
1.	Standard Plate count	Maximum 30,000 cfu/ml
2.	Coliform count	Absent in 1:10 dilution
3.	MBRT	More than 4 hours
4.	Alkaline phosphatase test	Negative

4.4 GRADING OF MILK

According to the Milk Ordinance and Code of the U.S. Public Health Service (1967), milk and certain milk products are graded on the basis of their bacterial counts for the following reasons:

It is widely accepted that the bacterial count of milk and certain milk products is an index of the sanitary quality. A high count does not necessarily mean that disease organisms are present, and a low count does not necessarily mean that disease organisms

are absent; but a high bacterial count does mean that the milk has come from diseased udders, or has been milked or handled under undesirable conditions, or has been kept warm enough to permit bacterial growth. This means, in the first two cases, that the chances of infection have been increased, and, in the last case, that any bacterial contamination which may have reached the milk has been permitted to increase to more dangerous proportions. In general, therefore, a high count means a greater likelihood of disease transmission. On the other hand, a wrong interpretation of the significance of low bacterial counts should be avoided, since low-count milk can be obtained from cows with brucellosis, or tuberculosis, or can have been handled by typhoid carriers or under unclean conditions.

When coliform organisms are present in pasteurized milk, they usually indicate that the milk has been contaminated after pasteurization. The phosphatase test is an index of the efficiency of pasteurization.

4.4.1 Collection of Samples

Samples for bacteriological examinations are collected by inspectors or other officials. They may be taken at random, and should be representative of the milk or milk products to be tested. In order to yield significant results, samples shall be taken while in the possession of the producer or of the plant, and must be collected in such a manner that they will fairly represent the condition of the milk as received at the plant intake or as finally delivered to the consumer.

At least 10 ml of well-agitated milk is collected and placed in a sterile sample bottle, which should be of such size that only about two-thirds of it is filled. This provides sufficient air space for vigorous agitation to ensure a uniform suspension of organisms before plating the milk. The sample, when taken, must be kept below 45°F; however, it may be kept above 45 but below 50°F when it is to be plated within 4 hr. It shall be protected against freezing.

Experience indicates that four or more samples should be examined before attempting to grade the supply. The samples should be taken from each supply during each 6-month grading period. The average bacterial plate count is expressed as the logarithmic average of the plate counts of the last four consecutive samples taken on separate days throughout the 6-month grading period.

4.4.2 Milk Standards

The number of organisms permissible in different grades of milk vary somewhat, depending upon standards set up by local public health authorities.

The highest grade of milk is known as certified milk, which is safeguarded at every step in its production, collection, and distribution. Milk collected under conditions not so carefully controlled is graded as A, B, or C. The ratings are based upon the bacterial count of milk and also upon the hygienic conditions under which it was produced. The standards of the various grades reported here are those set up by the U.S. Public Health Service milk ordinance (1967). They are as follows:

(I) Certified milk—raw—This is raw milk which conforms with the latest requirements of the American Association of Medical Milk Commissions in force at the time of adoption of this ordinance, and which is produced under the supervision of a medical milk commission reporting monthly to the health officer, and of the State health authority

or the municipal or county health officer. The usual standard is that the count must not go above 10,000 bacteria per millilitre.

All milk having counts in excess of this number must be placed in the following grades:

(I-A) Grade A raw milk for pasteurization—This is raw milk from producer dairies conforming with the following items of sanitation. The bacterial plate count or the direct microscopic clump count of the milk, as delivered from the farm, shall not exceed 100,000 per millilitre from an individual producer prior to commingling with other producer milk, and not exceeding 300,000 per ml. as commingled milk prior to pasteurization.

All milk for pasteurization shall be from herds which are located in modified accredited tuberculosis-free area, as determined by the U.S. Department of Agriculture, and which have been tested for tuberculosis not more than 6 years prior to the adoption of this ordinance and at least every 6 years after such test.

The health of the cow is a very important consideration because a number of diseases of cattle, including tuberculosis, brucellosis, Q fever, salmonellosis, staphylococcic infection, and streptococcic infection, may be transmitted to man through the medium of milk. The organisms of most of these diseases may get into the milk either directly from the udder, or indirectly through infected body discharges which may drop, splash, or be blown into the milk.

(I-B) Grade B raw milk for pasteurization—This is raw milk which does not meet the bacterial standard for Grade A raw milk for pasteurization, but which conforms with all other requirements. The bacterial plate count or the direct microscopic clump count of the milk, as delivered from the farm, shall not exceed 1 million per millilitre.

(I-C) Grade C raw milk for pasteurization—This is raw milk which does not meet the requirements for Grade B raw milk for pasteurization.

(II) Certified milk—pasteurized—This is certified milk (raw) which has been pasteurized, cooled, and bottled in a milk plant which conforms with the requirements for Grade A pasteurized milk.

(II-A) Grade A pasteurized milk—This is Grade A raw milk for pasteurization which has been pasteurized, cooled, and placed in the final container in a milk plant which conforms with certain items of sanitation. In all cases the milk shall show efficient pasteurization as evidenced by satisfactory phosphatase test, and at no time after pasteurization and before delivery shall the milk have a bacterial plate count exceeding 20,000 per millilitre, or a coliform count exceeding 10 per millilitre: Provided that the raw milk at no time between dumping and pasteurization shall have a bacterial plate count or a direct microscopic clump count exceeding 400,000 per millilitre.

(II-B) Grade B pasteurized milk—This is pasteurized milk which does not meet the bacterial-count standard for Grade A pasteurized milk, but which conforms with all other requirements for Grade A pasteurized milk. It has been made from raw milk for pasteurization of not less than Grade B quality, and has a bacterial plate count after pasteurization and before delivery not exceeding 50,000 per millilitre.

(II-C) Grade C pasteurized milk—This is pasteurized milk which does not meet the requirements for Grade B pasteurized milk.

4.5 STERILIZATION OF MILK

The shelf-life of pasteurized milk is not satisfactory in our country. Additionally it requires a cold chain after processing, which is rather costly and difficult to maintain. These limitations can be overcome by sterilization of milk. It ensures higher shelf-life even at ambient temperatures and increases convenience in shopping.

In India, according to PFA, the sterilization of milk means "heating milk continuously to a temperature of 115°C for 15 min or 145°C for 3 s or equivalent temperature-time combination to ensure preservation at room temperature for a period of not less than 15 days from the date of manufacture. Additionally, the sterilised milk shall show absence of albumin by negative turbidity test and it must be sold only in the container in which the milk was sterilised.

The last condition may not be proper in more commonly used method of sterilization called UHT (Ultra high temperature) which employs aseptic packaging after sterilization in a continuous plant.

Sterilized milk of satisfactory quality can only be produced if raw milk quality is good. The raw milk intended for sterilization should be (i) fresh, clean and free from off flavours, (ii) possess adequate heat stability, (iii) be free from abnormal milk (colostrum, late lactation, mastitis, etc.), and (iv) should have low numbers of both mesophilic and thermophilic spores. A treatment known as *Bactofugation* helps in reducing spore count in milk.

Bactofugation is a process to remove and reduce bacterial count of fluid milk by 99.98% by using mechanical forces. In the process, the milk is first pre-heated to reduce viscosity and then it is centrifuged in special machine (usually at 10,000 to 20,000 rpm) to remove microorganisms and especially spores. Subsequently the milk is pasteurized.

4.5.1 Methods for Sterilised Milk Preparation

There are three common methods for obtaining sterilised milk.

(i)   Complete-in-bottle system, wherein milk packed in bottles is heated in steam at 105-120°C for 15 to 30 min. This develops browning and gives cooked flavour to milk, hence not followed widely.

(ii)   UHT processing, wherein milk is heated (directly or indirectly) very rapidly upto a temperature of 130-150°C for few seconds. This does not give browning or cooked flavour, but gives higher bactericidal effect and hence it is very popular. This method is followed by aseptic packaging.

(iii)   Two-stage process, wherein sterilization of milk in bulk is first done by UHT process, followed by resterilizatin after filling bottles at 110-120°C for 15-20 min. This is not economical and not popular.

4.5.2 Basis of UHT Processing

The heat treatment necessary to kill microorganisms or their spores varies with the kind of organisms, their physiological state and the environment during heating. The term used to express the heat resistance of microorganism is "Thermal Death Time" (TDT), which is defined as the time necessary to kill all the organisms (or spores) under specified conditions at a specified temperature. The processing temperature-time combinations for UHT are based

on a TDT curve of some index organisms which are to be destroyed. *B. subtilis* from mesophilic type and *B. stearothermophilus* from thermophilic type are used as index organisms for fixing the temperature-time for UHT processing of milk. The combination is fixed to have a predetermined sterilization efficiency, which is measured in terms of number of decimal reductions (log cycle reductions) in the viable count of index organisms. This is designated as sterilizing effect which can be calculated as under:

$$\text{Sterilizing effect} \rightarrow = \log_{10} \frac{\text{Initial spore count / ml}}{\text{Surviving spore count / ml}}$$

$$= \log_{10} \text{Initial spore count/ml} - \log_{10} \text{surviving spore count/ml}$$

Example: If initial spore count in milk is 10,000/per ml and the final spore count in sterilized milk is 1/per 100 ml, then

$$\text{Sterilizing effect} = \log_{10} \frac{10,000}{0.01}$$

$$= 6$$

Generally UHT processing conditions for milk are designed to have sterilization effects of 12 for *B. subtilis* and 8 for *B. stearothermophilus.* In the temperature range of 120-150°C, the rate of destruction of spores is comparatively much higher than rate of browning of milk. Hence, the UHT processed milk remains whiter and has flavour similar to pasteurized milk.

4.5.3 Microflora of UHT Milk

When we call sterilised milk, literally it should be free from any living organism However, in actual practice it does not ensure 100% destruction of all living forms in milk, but it reduces it at such a low level that it satisfies our commercial interest of long shelf-life, and hence it is commonly called "Commercial sterility".

UHT milk may contain very low level of survivors belonging to thermophilic bacilli *(B. stearothermophilus)* or mesophilic bacilli *(B. subtilis, B. cereus,* Clostridia). These organisms are capable of causing sweet curdling and off flavours. However, of greater risk are the post heat treatment contaminants. They get entry usually from faulty filling and sealing of packs. They can be psychrotrophs, Enterococci, Bacillus, Micrococci or Streptococci and may bring about coagulation, proteolysis or flavour changes. Hence, great care is needed to check effective packaging. Generally, the standards set for spoilage are 1 container in 5000 in developed countries, while 1 Container in every 200 containers (0.5%) in India.

4.5.4 Tests and Standards for Sterilised Milk

As complete sterility is approached, the number of micro-organisms that survive becomes extremely small. Such a small number can be detected only when a huge volume of sample is examined. To make the analysis more practical, a pre-incubation technique is generally used. This technique was developed to increase the number of viable bacteria or spores, if any, by pre-incubating the sample at a suitable temperature for suitable time. The conditions usually recommended are 32, 37 or 55°C for 5, 7 or 10 days, respectively. Under these conditions, if the product sours or coagulates, the presence of viable organisms is

established. Complete asepsis is required, when a UHT product is analysed, because a single contaminant could confuse the entire situation.

BIS has set a very lenient standard of less than 5 spores per ml of sterilised milk. The sterility test, based on development of turbidity due to presence of albumin should be negative. Additionally it should not show variations exceeding 0.3 pH units or 0.02% titratable acidity during 7 days incubation at 55°C.

4.6 CLEANING AND SANITIZATION OF DAIRY PLANTS

Demand for improvement in the microbiological quality of milk and milk products have created a need for development of a better system for cleaning and sanitization of the product contact surfaces, because equipments alone can contribute about 60% of the total contamination.

Cleaning of dairy equipment implies the removal of 'soil' from the surface of each machine, while sanitization or sterilization implies the destruction of all pathogenic and all most all non-pathogenic microorganisms from equipment surface. Soil is the term used to mean material which has to be removed in a cleaning operation, which include milk residues, water deposits, detergent and sanitizer residues, dust, sediments or any other foreign matter left on equipment surfaces. Cleaning and sanitization are complementary processes needed to leave surfaces as free as possible from milk residues and viable organisms without adversely affecting the equipments.

Cleaning and sanitization process involves the following steps in sequence.

(i)   Draining
(ii) Pre-rinsing with tap water
(iii) Warm to hot detergent washing
(iv) Hot water rinsing
(v)   Sanitizing
(vi) Draining and drying

Detergents are the chemical compounds that are added to water to increase the ability of water to remove soil. Generally, in dairies, alkaline detergents with 0.15 to 0.60% alkalinity are used for cleaning. In more difficult cleaning operations, acids are also used. Sanitizer is a chemical or physical agent that is able to reduce the number of microorganisms in materials surfaces to a safe level. The types of sanitizers most commonly used in dairies are hot water, steam, chlorine compounds, iodophores or quaternary ammonium compounds.

Hand washing is the usual practice for small and easily accessible utensils/equipments. However, in bigger dairy plants, difficult to dismantle and reassemble, the popular method known as CIP (Cleaning-in-Place) is used. This is economical and time saving method too. In this method, the detergents and sanitizers are circulated in specific sequence and with required pressure and temperature, without dismantling the equipment.

Assessment of cleaning efficiency can be judged by visual inspection or staining method, which will indicate the presence of residual soil. However, the efficiency of sanitization can be tested by number of methods viz., swab test, rinse method, membrane filter technique, direct epifluorescent filter technique (DEFT), adhesive tape method and agar contact methods. Among these, swab test is vary popular, wherein a specified area of equipment is swabed in diluent (ringer's solution) and subsequently plated for total count.

Rinse method is also applied for utensils like milk bottles or cans, wherein the utensil is rinsed with a definite volume of dilution water, which is subsequently plated. Standards for judging the sanitary condition of utensils/equipment surfaces are given in table 4.2.

Table 4.2 : Microbiological standards for assessing the sterility of utensils/equipments as prescribed by BIS

	Rinse method Colony count per litre capacity of can	Swab method Colony count per 900 sq. cm. area of equipment surface
Satisfactory	< 1000	< 5000
Fairly satisfactory	1000 to 5000	5000 to 25,000
Unsatisfactory	> 5000	> 25,000

❑❑❑

5

CONCENTRATED MILKS

5.1 INTRODUCTION

Milk is perishable dairy product which requires proper cooling and handling to maintain its freshness and quality. Milk can be preserved for future use by various methods, the most common of which is concentration by removing water, using either heat or membrane methodology, followed by drying. The dehydrated milk has a longer shelf-life than the milk preserved by pasteurization and refrigeration.

Concentrated milk is the milk in which solids have been concentrated by removal of water. Water may be removed by evaporation, by sublimation or by partial freezing followed by removal of ice crystals. The first method is the most common. The higher concentration of solids which results, provides the product some protection against spoilage by certain chemical actions or microbial agents. The degree of this protection is closely related to the extent of concentration; the greater the proportion of the water removed, the more the protection. Whole milk, skim, or standardized milk are used in the preparation of concentrated milks. Partially *condensed* milk or *skim, sweetened* or *unsweetened* condensed milk or skims *evaporated* milk or skim, and *khoa* are concentrated milks. Prevention of Food Adulteration (P.F.A.) Act, Government of India uses the term "evaporated" to refer to unsweetened condensed milk or skim. Table 5.1 gives the composition of concentrated milks as per the P. F. A. act.

Table 5.1 : Composition of concentrated milks

| Product | Percentage of | | |
	Milk fat Min Max.	Total milk solids Minimum	Added cane sugar Minimum
Condensed milk unsweetend or evaporated milk	8.0 —	26.0	—

... contd.

Product	Milk fat Min Max.	Percentage of Total milk solids Minimum	Added cane sugar Minimum
Condensed milk Sweetened	9.0 —	31.0	40.0
Condensed skim unsweetened, or evaporated skim	— 0.5	20.0	—
Condensed skim, sweetened	— 0.5	26.0	40 .0

5.2 PROCESSING SCHEME FOR CONDENSED MILK

Bulk condensed milk may be manufactured using either whole or skim milk. Typically, the milk is pasteurized and then concentrated by heat in an evaporator until the product contains 40% to 45% total solids. Following concentration, the product may be dried or distributed for use as a concentrated milk. A detailed processing scheme for condensed milk is shown in Figure 5.1. Most condensed whole milk is used as an ingredient in the chocolate/confectionery, bakery, or dairy (frozen dessert) industries; condensed skim milk not subsequently dried is used primarily within the dairy industry (American Dairy Products Institute, 1996). These products are not considered to be commercially sterile and, when intended for shipment as an ingredient, they are immediately cooled and continuously held at temperatures below 7°C [45°F]. Microorganisms surviving the heat treatments usually are thermoduric or thermophilic types. Under proper handling and storage conditions, these organisms grow slowly, if at all, and are not expected to create keeping quality problems. If spoilage occurs, it usually is attributed to postheating contamination. Psychrotropic bacteria, yeasts, or molds may cause spoilage if product is held for unusually long periods or under improper storage conditions.

Raw Milk Receiving
Filter
Raw Milk Storage
Preheat
Clarifier/Separator
Pasteurization (161°F – 15 sec)
Evaporator System
Cold Storage
Filter
Condensed Milk Distribution
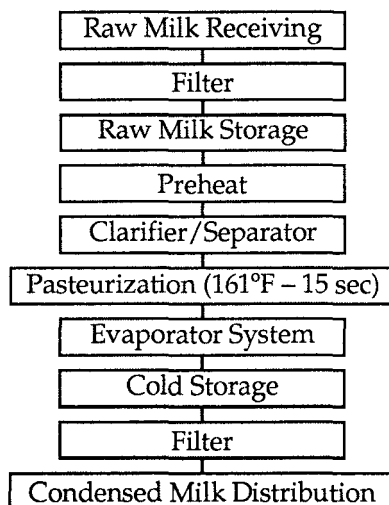
Fig. 5.1 : Processing scheme for condensed milk.

5.3 SWEETENED CONDENSED MILK

5.3.1 Historical Aspects

The father of Sweetened Condensed Milk was Gail Borden, school teacher, newspaper publisher, surveyor, port authority, and inventor. During a return voyage to the United States from the International Exhibition in London in 1851, Gail saw many babies dying as a result of the lack of pure, fresh milk. As a result, he resolved not only to find a way to preserve milk, but to keep its good taste and quality. After many unsuccessful attempts, he finally perfected his process and received a British patent on February 28, 1856. Shortly thereafter, he received a U.S. patent covering the same process on August 19, 1856. The same year, efforts were made to establish the first factory to produce the product in Wolcottville, Conn.; however, with meager resources at hand, he finally ran out of funds and the venture was a failure. The next year, in 1857, a second effort was made to establish a factory to produce the product in Burville, Conn.; however, the depression interfered with this effort. Finally, in 1858, with the backing of Jeramiah Millbank, a private banker in New York City, a successful business was established to produce and market Sweetened Condensed Milk.

The first product was peddled in the streets of New York City using a ladle out of a hand cart. In view of the fact that this was the first source of germ-free, wholesome milk, the demand increased rapidly. The name was changed from Gail Borden and Company to the New York Condensed Milk Company, and another plant was established in 1861. Shortly thereafter, with the advent of the Civil War, the Union Army commandeered all Sweetened Condensed Milk production for use by Union troops. Several more factories were built to keep up with the demand, and the military spread the product across the face of the United States during the Civil War. This was the beginning of the company, Borden, Inc., as we know it today.

Infant feeding instructions appeared on the label of the product from the early 1900's until 1938 when doctors started prescribing infant feeding formulas. The consumption of the product hit a peak during World War I when almost seven million cases were consumed during the year of 1919. The consumption of the product gradually declined after World War I and then again hit a peak shortly after World War II when sugar was very scarce to the consumer. Consumers were using the product in the preparation of desserts because of its high sugar content. Today, due to its unique physical properties, the product is used mainly in the preparation of fancy desserts.

These products are not popular in India.

5.3.2 Definition

Sweetened Condensed Milk is a mixture of pure, whole cow's milk and sugar with 60% of the water removed before it is packed in hermetically sealed cans. An approximate analysis of the product is as follows:

	(%)
Milk fat	8.5
Milk-solids-not-fat (MSNF)	20.5
Sugar	44.0
Moisture	27.0
Total	100.0

Sweetened Condensed Milk is actually very similar to homemade jelly, except that sugar is being used to preserve milk solids rather than the juice of fruits or berries, as in the case with jelly.

The primary difference between condensed and sweetened condensed milks is the addition of sugar. Sweetened condensed milk is preserved by addition of sugar, which increases the osmotic pressure to a point inhibitory to most microorganisms. The increased milk solids content also increases the osmotic pressure. The sugar-in-water concentration of sweetened condensed milk is called the "sugar ratio," which is calculated as follows:

$$\frac{\% \text{ sugar in condensed milk}}{100 - \text{total milk solids in condensed milk}} \times 100 = \text{sugar ratio}$$

5.3.3 Manufacturing Process

Raw milk arrives fresh from the farm at the condensory's receiving platform where it is checked for quality and temperature. The milk is tested for fat and total solids and then standardized. This standardized milk is blended with sugar and pumped into large stainless steel tanks called "hot wells" to pasteurize the milk and destroy any pathogenic bacteria that might be present. The heat treatment in the hot well is varied with the seasons of the year to control physical instability which otherwise might occur in the product.

The blend of heated, standardized whole milk and sugar is then drawn into a vacuum condensing pan to reduce it to the required consistency and total solids content. To preserve the original flavour and colour, the milk is boiled at a low temperature of approximately 54°C under vacuum. When the correct concentration is reached, the milk is drawn quickly from the vacuum pan to the vacuum cooling tank where the temperature is rapidly lowered to approximately 21°C. During this cooling procedure, a predetermined amount of finely ground milk sugar (lactose) is added to the product to control the crystallization of sugar present. At this point, the preparation of the product is completed. It is now thick, smooth in texture, and creamy in colour. Until canned, the milk is held in insulated storage tanks that are sealed and sterile.

Before filling, the consumer size cans are washed and sterilized inside and out by live steam and then fed automatically into a filling machine that is protected to prevent bacterial, yeast, and mold contamination to the product. The milk flows from the storage tank to a continuous filling machine where the filled cans are immediately sealed. Unlike most canned foods, the container is completely filled to prevent the presence of air which could result in the growth of yeast and/or mold in the product. After the cans are coded with the factory date of manufacture, they are then labeled and finally packed for shipping, 24 cans to a case. When final approvals for bacteriological, chemical, and physical analyses are given, the milk is shipped out to supermarkets across the United States and around the world.

Today, most of the Sweetened Condensed Milk in the United States is still produced by Borden, Inc., and the product is still essentially the same as that produced by Gail Borden back in 1856. The product is packed in 14-oz cans and is used in the preparation of fancy desserts. Also, a considerable amount of bulk product is produced for use in the baking and confectionery industries.

5.3.4 BIS Standards for Sweetened Condensed Milk

Sweetened condensed milks are generally tested for total bacteria, yeast and mold counts. Microbiological standards specified by BIS are given as follows:

(A)	Bacterial count (maximum)	500 cfu/g
(B)	Coliforms	Negative
(C)	Yeast and Mold count (maximum)	10 cfu/g

5.4 EVAPORATED MILKS

5.4.1 History

Evaporated milk, like other processed canned foods, originated with the experiments of the French scientist Nicholas Appert (Flake and Clark, 1991). Appert, whose work on food preservation began in 1795, was the first person to evaporate milk by boiling it in an open container and then preserving it by heating the product in a sealed container. Fifty years later, another French scientist, Louis Pasteur, laid the scientific foundation for heat preservation through demonstrations that food spoilage could be caused by bacteria and other microorganisms.

Patents dealing with preservation of milk after evaporation in a vacuum were granted to Gail Borden by the United States and England in 1856. These patents applied to concentrating milk without addition of sugar. In 1884, U. S. patent number 308,421 was issued for "an apparatus for preserving milk" and, in 1885, the first commercial evaporated milk plant in the world was opened in a converted wool factory in Highland, IL where "evaporated cream" was manufactured and sold (Flake and Clark, 1991).

5.4.2 Products and Processing

Evaporated milk is a canned whole milk concentrate to which a specified quantity of vitamin D has been added and to which vitamin A may be added. It conforms to the Food and Drug Administration (FDA) Standard of Identity 21 CFR 131.130 (U. S. Department of Health and Human Services, 1995a), having a minimum of 6.5% milk fat, 16.5% milk solids-not-fat, 23% total milk solids, and 25 IU vitamin D per fluid ounce. Related evaporated milk products are evaporated skimmed milk, evaporated low-fat milk, evaporated filled milk, and evaporated goat's milk. Evaporated skimmed milk conforms to the FDA Standard of Identity 21 CFR 131.132 (U. S. Department of Health and Human Services, 1995a) and contains not less than 20% of total milk solids, not more than 0.5% milk fat, with added vitamins of 25 IU vitamin D and 125 IU vitamin A per fluid ounce. Standards of Identity have not been established for the other evaporated milk products. Their typical compositions are the following:

Evaporated low-fat milk—2% milk fat, 18% non-fat milk solids, vitamins A and D added

Evaporated filled milk—6% vegetable fat, 17.5% non-fat milk solids, vitamins A and D added

Evaporated goat's milk—not less than 7% milk fat and 15% non-fat milk solids, vitamin D added

A typical processing scheme for evaporated milk (Fig. 5.2) begins with high-quality, fresh whole milk, to which vitamins, emulsifiers, and stabilizers are added. The product is then pasteurized, concentrated under reduced pressure in an evaporator, homogenized, cooled, and standardized to the composition desired in the final product. After cans are filled and sealed, they are sterilized in a three-phase continuous system consisting of preheater, retort, and cooler, then labeled and packed for shipment. In the United States, evaporated milk is packed in 5-, 12- and 97-fluid ounce lead-free cans. In 1995, production of evaporated milk and related products (evaporated skimmed milk, evaporated low-fat milk, and evaporated filled milk) was slightly more than 498 million pounds (American Dairy Products Institute, 1996).

Evaporated milk processing is covered by FDA regulations dealing with thermally processed low-acid foods packaged in hermetically sealed containers (U. S. Department of Health and Human Services, 1995). Therefore, manufacturers of evaporated milk and related products must comply with stringent processing regulations, including the establishment and filing of scheduled processes with FDA and the maintenance of strict processing records.

Raw Milk Receiving

Raw Milk Storage

Balance Tank

Separator/Clarifier

Vitamins → Blend Tank ← Stabilizer / Emulsifier

Pasteurization
(161°F – 15 sec)

Evaporator System

Homogenizer

Cooler

Dry/Condensed Dairy Ingredients → Standardize — Filter

Evaporated Storage

Filter

Filter

Retort
(242 – 250°F 10-15 min)

Label/Case

Storage

Evaporated Milk
Shipping
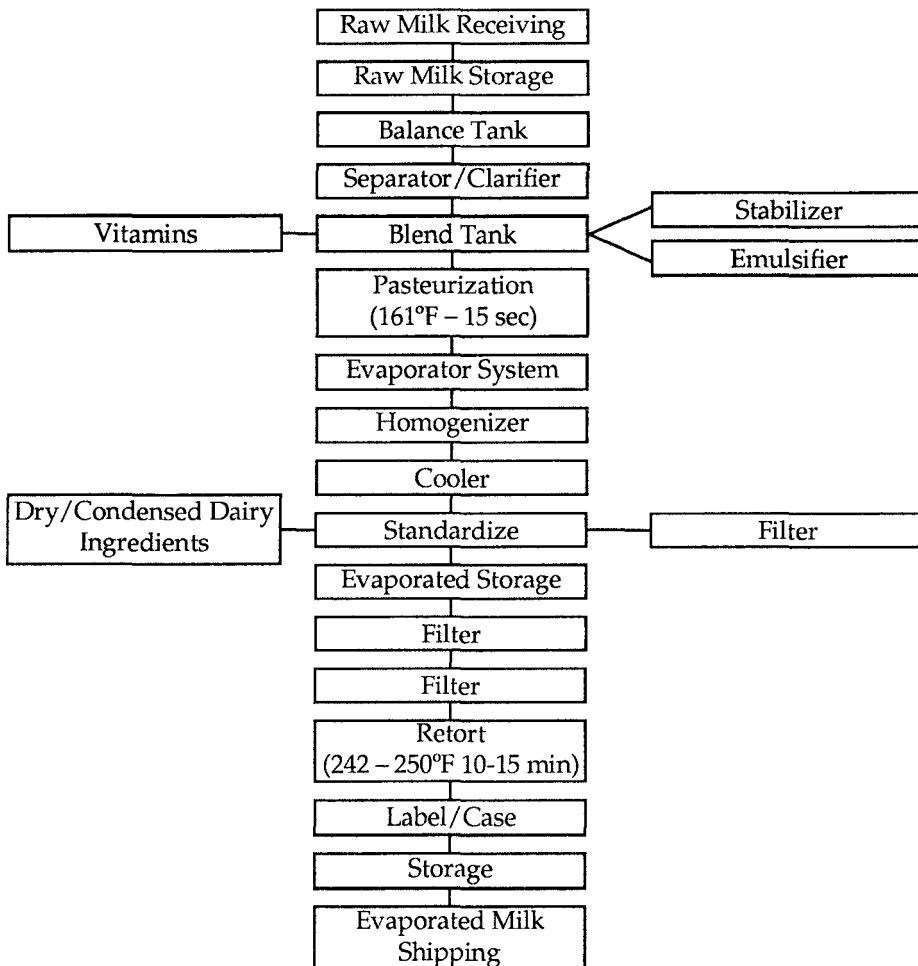
Fig. 5.2 : Processing scheme for evaporated milk.

5.4.3 Microbiology

Because of the heat processes and packaging used in the manufacture of evaporated milks, the product is commercially sterile. This means that the product is free of all microorganisms of public health significance and does not show microbial defects during its intended shelf-life under normal conditions of handling, storage, and distribution. Whereas vegetative cells do not survive evaporated milk processing, and absolute sterility is obtained in most cans, small numbers of nonpathogenic spores occasionally may survive the heat treatment and, depending on the microorganism and its previous growth and heat exposure, subsequently may germinate (Curran and Evans, 1945). Kalogridou-Vassiliadou (1992) studied 40 strains of bacilli implicated in causing flat sour spoilage in evaporated milk. The microorganisms were identified as *Bacillus stearothermophilus* (five strains), *Bacillus licheniformis* (10 strains), *Bacillus coagulans* (15 strains), *Bacillus macerans* (five strains), and *Bacillus subtilis* (five strains). Species of the genus *Bacillus* (i.e., *cereus, coagulans, megatherium, stearothermophilus,* and *subtilis*) earlier were implicated in cases of evaporated milk spoilage (Foster *et al.*, 1957; Hammer and Babel, 1957). Recently, Langeveld *et al.* (1996), in studies of *Bacillus cereus* naturally present in raw milk, reported no evidence that this organism would cause intoxication in healthy adult humans at levels less than 10^5 mL. Classic studies (Curran and Evans, 1945; Theophilus and Hammer, 1938) on the microbiology of evaporated milk have contributed significantly to the knowledge of the microbiology of this product.

Under current continuous processing conditions wherein heat treatments of 117°C to 121°C (242°F—250°F) for 10 to 15 minutes are common and batch retorting is uncommon, spoilage of evaporated milk is unlikely to be encountered. Specific methods for the microbiological examination of evaporated milk are contained in *Standard Methods for the Examination of Dairy Products* (Marshall, 1992).

□□□

6

DRIED MILKS AND
DRY WHEY PRODUCTS

6.1 INTRODUCTION

The milk can be preserved by dehydrating to various degrees. The dehydrated milk has a longer shelf-life than the milk preserved by Pasteurization and refrigeration. Milk from which all of water has been withdrawn is called dried milk or milk powder.

Powdered milk is made by the removal of most of the water from milk so that the milk solids are accompanied by not more than 5%, perhaps as little as 2% moisture. Water is removed in two stages. In the first stage the milk is concentrated by vacuum evaporation to about one-third of its original value. In the second stage the milk is dried, usually on a roller drier or in a spray drier. Three classes of milk powder are recognised by P. F. A. Act. They are milk powder, skimmed milk powder, and partially skimmed milk powder. The principal differences are in their final composition, and are influenced by the class of milk which is dried (Table 6.1).

Table 6.1 : Composition of several dried milks

| Powder | Product composition in percentage | | | |
	Fat	Solid non-fat	Water	Ratio Fat/Solid non-fat
Whole milk	26.0 to	66.5 to	3.7 to	1:2.3 to
	28.5	70.3	5.0	1:2.7
Skim milk	1.3 to	93.5 to	3.5 to	1:59.3 to
	1.6	94.9	5.0	1:62.3
Partially	8.5 to	71.0 to.	4.2 to	1:3 to
skimmed milk	24.0	86.5	5.0	1:10.2

6.2 HISTORY

The development of the dry milk industry stems from the days of Marco Polo in the 13th century. It is reported that Marco Polo encountered sun-dried milk on his journeys through Mongolia and that, from this beginning, dry milk products evolved (Clark, 1991). Through early pioneering scientists, such as Appert and Borden, the basic methods were developed for emergence of processes for drying milk products. Ekenberg and Merrill have been acknowledged as the developers of the first commercial roller-and spray-process drying systems, respectively, in the United States (Beardslee, 1948). Since the initial development of commercial drying systems, significant technological advances have been made, resulting in the manufacture of a variety of dry milk products.

6.3 DRY MILK PROCESSING

Fluid milk typically undergoes the following handling and processing steps before being dried (Fig. 6.1)

(A) Receipt of good quality fluid milk by the processing plant.

(B) Separation of fluid milk, if non-fat dry milk is to be produced, with the resulting cream processed into butter. For the manufacture of dry whole milk, separation is omitted but often it is replaced by clarification.

(C) Pasteurization by high temperature-short time processes which heat the milk to 71°C and maintain it at that temperature continuously for 15 sec.

(D) Dependent upon intended use of the final dry milk product, especially non-fat dry milk, an additional heat treatment may be utilized to obtain product with a specific undenatured whey protein nitrogen content.

(E) Condensing of the milk in an evaporator or vacuum pan. Because moisture is removed more efficiently by this process than by heated air within the dryer, the total solids content of the condensed milk is increased to at least 40%.

Complete and detailed discussions of the techniques utilized in processing fluid milks into dry milk products have been presented by Hall and Hedrick (1971) and Webb *et al.* (1974).

Since the first commercial applications, dry milks have been produced by basically two processes—roller (drum) and spray techniques. Many advances in engineering design and equipment fabrication have been noted since 1900 until the present milk drying processes which can best be described as follows:

(A) Roller Process—Two large rollers, usually steam heated internally and located adjacent and parallel to each other, revolve at a desired speed. The rollers revolve in opposite directions, contacting a reservoir of either pasteurized fluid or condensed milk. During rotation, the fluid milk product dries on the hot roller surface; after approximately ¾ of a revolution, a carefully positioned, sharp stationary knife detaches the milk product, now in the form of a thin dry sheet. The dry milk next is conveyed by an auger to a hammermill where it undergoes a physical treatment to convert it into uniformly fine particles which then are packaged, usually in 50- or 100-lb bulk packages.

(B) Spray Process—Two basic configurations of spray dryers presently are in use, these being horizontal (box) and vertical (tower) dryers. In both, pasteurized fluid milk which has been condensed to a total solids of 40% or above is fed under pressure to a spray nozzle, or atomizer, where the dispersed liquid comes into contact with a current of filtered, heated

air. The droplets of condensed milk are dried almost immediately and fall to the bottom of the fully enclosed, stainless steel drying chamber. The dry milk product is removed continuously from the drying chamber, transported through a cooling and collecting system, and finally conveyed into a hopper for packaging in 50- or 100-lb bags, or in Tote bins. Collecting systems most commonly used in conjunction with spray dryers are either cyclones or bag units.

Recently, sanitary standards have been formulated for milk products spray drying systems (3-A Sanitary Standards Committees 1971). These standards relate to the sanitary aspects of equipment and establish specific criteria for design, material, fabrication, and air supply and quality.
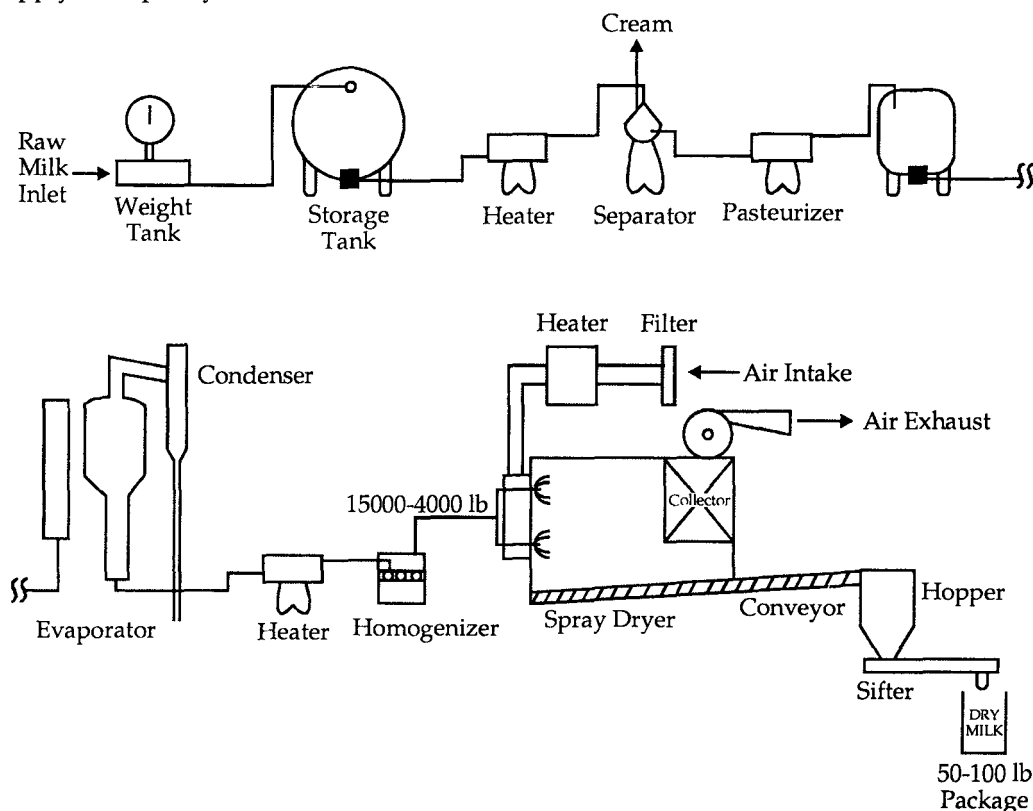
Fig. 6.1 : Flow diagram for dry milk processing.

6.4 INSTANT DRY MILKS

Instant dry milks are dry milk products that have been produced in such a manner as to substantially improve their dispersing and reliquefication characteristics. In the United States, both single-pass and agglomerated instant dry milks are manufactured, the latter comprising the majority of instant dry milk. In the process of manufacturing agglomerated instant, the surface of warm dry milk particles is re-wetted permitting particles to coalesce upon contact with each other, thus forming large particles or clusters. Following the agglomeration step, the dry milk is redried and recooled, perhaps sized, and packaged.

Figure 6.2 shows one type of instantizer for agglomerating dry milks.

EXHAUST BLOWER
OUT OF DOORS

PRODUCT

ALTERNATE INLETS

A

SIGHT GLASS

D

DUST
COLLECTOR

SAG DUMP HOPPER

C

VARIABLE SPEED

SIFTER

VARIABLE
SPEED

S

ROLL FILTER

SLENDER

BAG FILTER

STEAM IN

DRYING OVEN
(GAS FIRED)

COOLING COILS

TO TOTE BINS
&
PACKAGING

RECYCLE OF FINES

PRODUCT

OVEN

ABSOLUTE FILTER

CLEAN AND DUCT

SURGE
HOPPER

A = AGGLOMERATING TUBE

D = DRYING SHAKER BED

C = COOLING SHAKER BED

S = SIZING ROLLS WITH SHAKER SCREEN

AIR LOCK

BLOWER

COURTESY OF CHERRY-BURELL CORP

Fig. 6.2 : Schematic diagram of a dry milk instantizing system.

6.5 COMPOSITION OF DRY MILK PRODUCTS

The average composition and food value of non-fat dry milk, dry whole milk and dry butter milk are shown in Table 6.2. In addition to the nutrients shown in this table, dry milks contain many other vitamins and minerals. Fat-soluble vitamins are known to be present in appreciable amounts in dry whole milk, in smaller amounts in dry buttermilk, and in only minor quantities in non-fat dry milk. Among the water-soluble vitamins, B12 is furnished in significant amounts by dry milks, and approximately 27 mg of vitamin C (ascorbic acid) are furnished per pound of dry whole milk. Important amounts of minerals such as sodium, potassium, and magnesium also are supplied by dry milks.

Table 6.2 : Approximate composition and food value of dry milks

Constituents	Nonfat Dry Milk	Dry Whole Milk	Dry Buttermilk
Protein (N × 6.38) (%)	36.0	26.0	34.0
Lactose (Milk Sugar) (%)	51.0	38.0	48.0
Fat (%)	0.7	26.75	5.0
Moisture(%)	3.0	2.25	3.0
Minerals (ash) (%)	8.2	6.0	7.9
Calcium(%)	(1.31)	(0.97)	(1.3)
Phosphorus (%)	(1.02)	(0.75)	(1.0)
Energy (cal/lb)	1630.0	2260.00	1700.0

Source: American Dry Milk Institute.

6.6 STANDARDS FOR DRY MILK PRODUCTS

Industry microbiological standards for dry milk products are established by the American Dairy Products Institute. In addition, government standards for these products also have been generated by the USDA and the FDA (U. S. Public Health Service, 1995). Table 6.3 shows these standards by source, product, and, as applicable, grade.

Table 6.3 : Microbiological standards for dry milk products[a]

Product	American Dairy Products Institute standards[b]	United States Department of Agriculture standards[b]	Food and Drug Administration (grade A) standards
Non-fat dry milk			
Extra grade	SPC: 40,000/g Coliform: 10/g	SPC: 40,000/g Coliform: 10/g	Bacterial estimate: 30,000/g Coliform: 10/g
Standard grade	SPC: 75,000/g Coliform: 10/g	SPC: 75,000/g Coliform: 10/g	

... contd.

Product	American Dairy Products Institute standards[b]	United States Department of Agriculture standards[b]	Food and Drug Administration (grade A) standards
Dry whole milk			
Extra grade	SPC: 50,000/g Coliform: 10/g	SPC: 50,000/g Coliform: 10/g	None
Standard grade	SPC: 100,000/g Coliform: 10/g	SPC: 100,000/g Coliform: 10/g	
Dry buttermilk			
Extra grade	SPC: 50,000/g Coliform: 10 g	SPC: 50,000/g Coliform: 10/g	Bacterial estimate: 30,000/g Coliform: 10/g
Standard grade	SPC: 200,000/g Coliform: 10 g	SPC: 200,000/g Coliform: 10 g	

[a]*All counts expressed as "not more than."*

[b]*DMC may not exceed 100 million/g for ADPI- and USDA-graded non-fat dry milk and dry whole milk.*

Abbreviations: DMC, direct microscopic clump count; SPC, standard plate count.

The standards recommended by BIS (Bureau of Indian Standards) are mentioned in Table 6.4. However, as per PFA, the WMP (Whole milk powder), SMP (Skim milk powder) or Infant milk food should not contain more than 50,000 cfu/g for total bacteria, while coliforms should be absent in 0.1 g sample.

Table 6.4 : Microbiological standards for milk powders as recommended by BIS

	Types	WPM and extra-grade SMP	Standard Grade SMP
(1)	Total bacterial count (maximum, cfu/g)	40,000	50,000
(2)	Coliform count	Absent in 0.1 g	Absent in 0.1 g
(3)	*Salmonella*	Absent in 25 g	Not specified
(4)	*Staph. aureus* (Coagulase positive)	Absent in 0.1 g	Not specified
(5)	*Shigella*	Absent in 25 g	Not specified

6.7 MICROBIOLOGY OF DRY MILKS

Relatively few species of bacteria have been reported as being naturally occurring in dry milks. Hammer and Babel (1957) and Foster *et al.* (1957), in earlier texts covering the microbiology of dry milk products, summarized literature reports indicating microorganisms of the genera *Streptococcus, Micrococcus, Bacillus, Clostridium,* and *Sarcina* as comprising the

primary microflora of dry milks. Rodriquez and Barrett (1986), based on a study of the microbial population and growth in reconstituted dry milk, confirmed the occurrence of viable cells of the genera *Bacillus* and *Micrococcus* in non-fat and dry whole milks.

Since initiation of the requirement that all milk be pasteurized before drying, current heat treatments used to process dry milks destroy all microorganisms of public health significance. Relatively low numbers of microorganisms survive the processing, and those heat-resistant organisms (both spore-forming and non-spore-forming types) rarely, if ever, are responsible for finished product deterioration. Because the drying process is accomplished in a completely closed system, post-processing contamination also is rare. When such occurs, it usually is from an airborne source. Because of the low moisture levels in dry milks, those viable organisms that may be present are unable to grow and decrease in number during storage. Specific methods for the microbiological examination of dry milks are contained in *Standard Methods for the Examination of Dairy Products* (Marshall, 1992).

Spray-dried milks have been implicated in outbreaks of staphylococcal food poisoning (Anderson and Stone, 1955; Armijo *et al.*, 1957). In both instances, the illnesses were caused by a preformed enterotoxin that was not inactivated by the drying process. Miller *et al.* (1972), in a study of the effect of spray drying on survival of *Salmonella* and *Escherichia coli*, reported that heat treatments typically associated with spray drying could not be counted on to supplant adequate pasteurization and post-drying sanitary procedures. Bradshaw *et al.* (1987), in studies of the thermal resistance of disease-associated *Salmonella typhimurium* in milk, reported the organism did not survive pasteurization.

Doyle *et al.* (1985) studied survival of *Listeria monocytogenes* during the manufacture and storage of non-fat dry milk. Concentrated (30% solids) and unconcentrated skim milks were inoculated with 10^5 to 10^6 *L. monocytogenes*/mL. They reported reductions of 1 to 1.5 $\log_{10}$ *L. monocytogenes*/g occurred during the spray drying process and that the organism progressively died during storage. The inoculated milks were not pasteurized before drying. Bradshaw *et al.* (1985) and Donnelly *et al.* (1987) reported that *L monocytogenes* did not survive in milk during pasteurization. Earlier studies (Nichols, 1939; Higginbottom, 1944) also reported on the destruction of microorganisms during drying and the fate of surviving organisms during storage.

6.8 DRY WHEY PRODUCTS

6.8.1 History

Although spray and roller processes have been used to dry whey for many years, development of a whey processing industry in the United States did not fully materialize until the organization of the Whey Products Institute in 1971 (Clark, 1991b). At that time, development of product identity and quality standards was undertaken as a guide to production of uniformly high-quality whey products. In 1981, the FDA accepted industry-recommended common and usual names for a variety of whey products and affirmed the generally recognized as safe (GRAS) status of these products and their method of manufacture (U. S. Department of Health and Human Services, 1981). Technological changes associated with whey processing are dynamic. In no area of the modern dairy industry have changes of a technical nature been as innovative and rapid as in the whey products segment.

Important applications to whey processing include use of selective membrane techniques that allow the various whey constituents to be separated into protein-, carbohydrate-, or mineral-rich streams, which then may be further processed and made available in concentrated functional forms. Significant developments, reflecting continuing changes, are anticipated in this area.

6.8.2 Products

The primary whey products currently manufactured in the United States are concentrated and dry whey, and the modified whey products, including reduced-lactose whey, reduced-minerals whey, and whey protein concentrate. Other modified whey products manufactured in smaller quantities include lactalbumin (minimum protein content, 80%) and whey protein isolate (minimum protein content, 90%). Lactose, the carbohydrate of milk, also is being produced in large quantities as a co-product with the manufacture of modified wheys. Table 6.5 defines the commonly known whey products currently being manufactured.

Table 6.5 Composition of Whey Products[a]

Name of product	Major parameters (%)[b]				
	Protein	Fat	Ash	Lactose	Moisture
Whey	10-15	0.2-2.0	7-14	61-75	1-8
Concentrated whey	10-15	0.2-2.0	7-14	61-75	1-8
Dry or dried whey	10-15	0.2-2.0	7-14	61-75	1-8
Reduced-lactose whey	16-24	0.2-4.0	11-27	60 max	1-6
Reduced-minerals whey	10-24	0.2-4.0	7 max	85 max	1-6
Whey protein concentrate	25 min	0.2-10.0	2-15	60 max	1-6
Lactose	N/A	N/A	0.3	98 min	4-6

[a]*FDA affirmation of direct food substances as generally recognized as safe.*
[b]*On a dry product basis.*

6.8.3 Processing

A typical processing scheme for the manufacture of dry whey is shown in figure 6.3. Some whey drying operations receive only condensed whey for processing; others receive condensed and fresh fluid whey. The solids concentration of the transported condensed whey and the time-temperature conditions of its shipment determine how the product is processed before entering the drying system. Currently, the USDA requires all condensed whey containing less than 40% solids to be pasteurized or re-pasteurized in the processing plant where it is to be dried. The process of drying is similar to that used to manufacture dry milks and some processing plants may dry both products interchangeably.

Processing operations to manufacture modified whey products include reverse osmosis, ultrafiltration, and electrodialysis procedures, some of which may be proprietary in nature. For more information on these processes, various published texts (Sienkiewicz and Riedel, 1990; Gillies, 1974) may be consulted.

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                    ┌─────────────────────┐
                    │ Raw Whey Receiving  │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │      Clarifier      │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │      Separator      │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │  Raw Whey Storage   │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │       Preheat       │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │   Pasteurization    │
                    │  (161°F - 15 sec)   │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │ Concentration System│
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐  ┌────────────┐  ┌────────┐
                    │   Drying System     │──│ Heated Air │──│ Filter │
                    └─────────────────────┘  └────────────┘  └────────┘
  ┌────────────┐  ┌──────────┐  ┌─────────────────────┐
  │ Cooling Air│──│  Filter  │──│      Cooling        │
  └────────────┘  └──────────┘  └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │    Bulk Storage     │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │       Sifter        │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │      Packaging      │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │       Storage       │
                    └─────────────────────┘
                               │
                    ┌─────────────────────┐
                    │  Dry Whey Shipping  │
                    └─────────────────────┘
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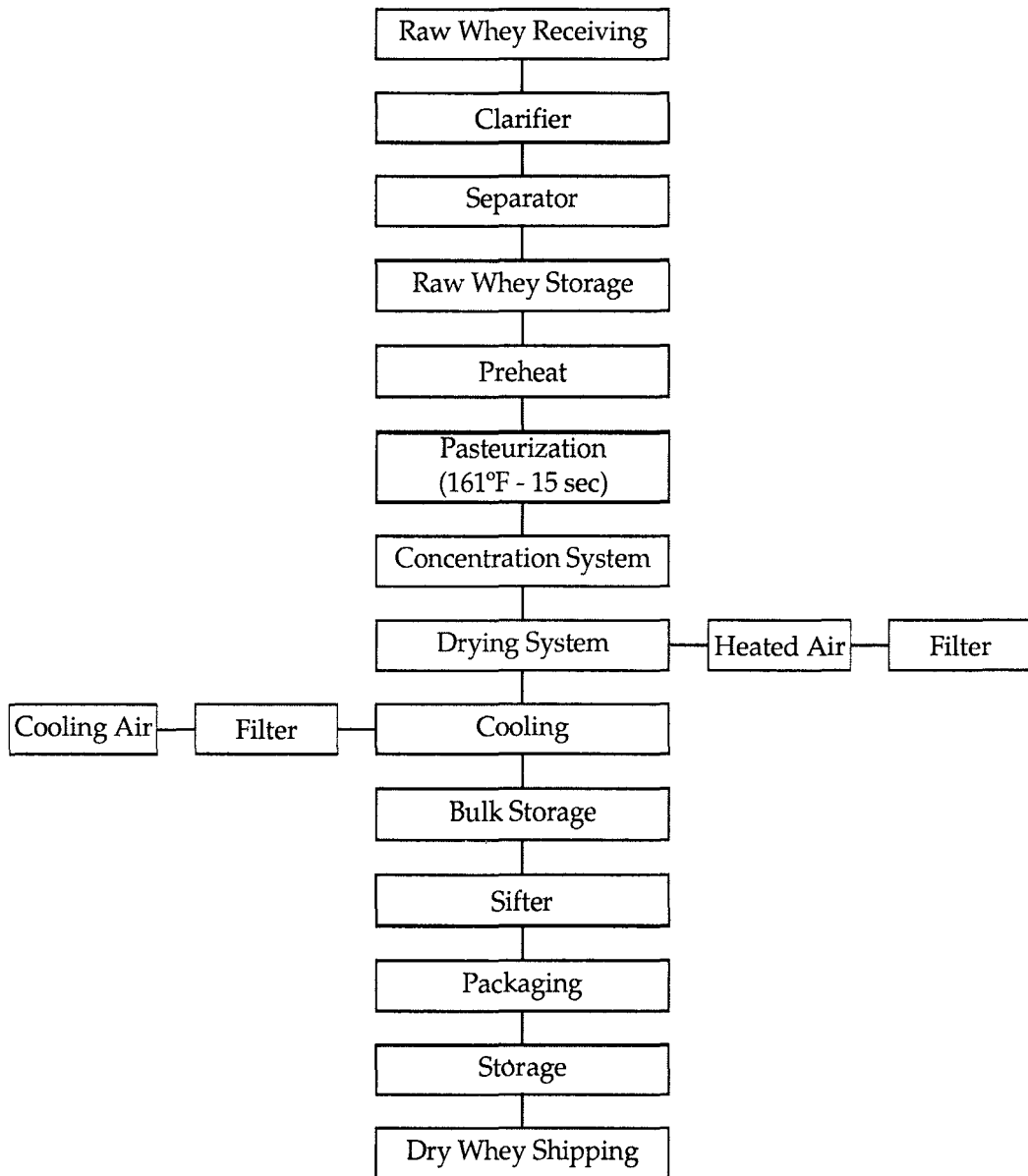
Fig. 6.3 : Processing scheme for manufacture of dry whey.

6.8.4 Standards

As for dry milk products, industry microbiological standards for whey products have been established by the American Dairy Products Institute, the USDA, and the FDA. Table 6.6 shows current microbiological standards for whey products.

Table 6.6 : Microbiological standards for whey products[a]

Product	American Dairy Products Institute standards	United States Department of Agriculture standards	Food and Drug Administration (grade A) standards[b]
Condensed whey	None	None	Bacterial estimate: 30,000/g Coliform: 10/g
Dry whey			
Extra grade	SPC: 50,000/g Coliform: 10/g	SPC: 50,000/g Coliform: 10/g	Bacterial estimate: 30,000/g Coliform: 10/g

[a]All counts expressed as "not more than."

[b]includes grade A dry whey and dry whey products.

Abbreviations: SPC, standard plate count.

6.8.5 Microbiology

As drying processes for whey are essentially the same as those for milk, the discussion of dry milk microbiological considerations also apply to dry whey. Microbiological methods to assay the quality of whey products are contained in *Standard Methods for the Examination of Dairy Products* (Marshall, 1992).

Cultural or direct microscopic (DMC) procedures may be used. If using the latter, it must be understood that most whey processed is derived from cheese manufactured using bacterial cultures; thus, large numbers of viable lactic organisms are present in fresh whey. Except for the more heat-resistant strains of lactic bacteria, these organisms are not expected to survive pasteurization and are not detected by cultural techniques. However, when freshly dried whey is examined by direct microscopic techniques, cells of nonviable bacteria often stain. Therefore, results of DMC techniques used to assess the quality of dry whey must be interpreted with care.

Merin (1986), in a study of the microfiltration of whey using 1.2-μm pore size membranes, reported that the membranes reduced bacterial counts by one to three times and that increased fat content in the feed stream governed the decrease. Fat trapped on the membrane formed a barrier to microorganism penetration into the permeate.

□□□

7

MICROBIOLOGY OF STARTER CULTURES

7.1 INTRODUCTION

The production of fermented dairy products, that is, cheese, yogurt, butter and others, depends greatly upon the activity of the lactic acid bacteria which are used as starter cultures. These bacteria are responsible for the hydrolysis of milk proteins, lactose, and citrate and the production of acid and flavour compounds, as well as other characteristics of the end-product.

A starter culture is any active microbial preparation intentionally added during product manufacture to initiate desirable changes. These microbial preparations can consist of lactic acid bacteria, propioni bacteria, surface ripening bacteria, and yeasts, as well as molds.

Starter cultures have a multifunctional role in dairy fermentations. Their ability to rapidly produce acids aids in separation of curd from whey during cheese manufacture, modifies texture of cheese and cultured milks, and enhances preservation. Production of low molecular weight compounds such as diacetyl contributes to flavour and aroma. Gas production can cause eye formation in cheese. Development of flavour and changes in texture during ripening of cheeses is associated with enzymes originating from bacterial and fungal cultures, depending on the cheese variety.

Starter cultures can be defined as "the selected groups of pure and actively growing microorganisms, which are used singly or in combination of inoculum to bring about desirable changes in the medium (milk) to form the finished product."

This chapter discusses various microbiological aspects of starter cultures used in dairy industry.

7.2 FUNCTIONAL ROLE OF STARTER CULTURES

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 flavour production. In addition to these, the starters, also help in imparting pleasant acid taste, conferring protection against potential pathogens and providing a longer shelf life to the product.

The foremost requirement for carefully regulated acid production by starter bacteria can be understood when the role of acidity is considered during the preparation of fermented foods. These bacteria chiefly convert approximately 1% of lactose in milk to lactic acid with very little by-products such as acetic acid, ethanol, CO_2 etc. and normally with no substances causing taints and hence leading to a clean and wholesome milk souring.

Functions of starter cultures in dairy industry are summarized as follows:

1. Produce lactic acid.
2. Bring about coagulation.
3. Produce volatile flavour compounds like diacetyl, acetaldehyde etc.
4. Possesses controlled proteolytic and lipolytic activity.
5. Produce other compounds like CO_2, alcohol, propionic acid etc. which are essential in making dairy products like kefir, Swiss cheese etc.
6. Control growth of spoilage organisms and pathogens.
7. Give health benefits (Probiotic).
8. Help in texturizing and ripening of cheese.

The first use of starter culture was made by Storch and Conn in 1890 for developing flavour and aroma in butter. The first use of starter for cheese making in Britain was made in 1895. Starters were introduced in England by J. Stubbs in 1905. Commercial cultures were placed on the market by the Dannish farm of Hansen towards the end of nineteenth century. Today this is one of the most advanced laboratory, known as Chr. Hansen's Lab in the world, supplying dairy cultures.

7.3 NATURE OF STARTERS

The most commonly used starter cultures in dairy industry include lactococci, lactobacilli, leuconostocs, pediococci and several other types. Some common bacterial cultures used in the manufacture of a variety of fermented foods throughout the world are listed in Table 7.1 alongwith the possible role they play in the final product.

Table 7.1 : Common starter cultures used for preparation of fermented dairy products and their role

Starter culture	Role/function	Fermented dairy product
Lactococcus lactis subsp. lactis	Acid	Cultured butter milk, sour cream, cottage cheese, all types of cheeses (domestic and foreign), starter culture etc.
Lactococcus lactis subsp. cremons		
Lactococcus lactis subsp. diacetylactis	Acid and flavour	Sour cream, ripened cream, butter, cheese, butter milk, starter cultures etc.
Streptococcus thermophilus	Acid	Emmental, Cheddar and Italian cheese and yoghurt

... contd.

Starter culture	Role/function	Fermented dairy product
S. durans, S. faecalis	Acid and Flavour	Soft Italian, Cheddar and some Swiss cheese varieties
Leuconostoc citrovorum, Leuco. dextranicum	Flavour	Cultured butter milk, sour cream, cottage cheese, ripened cream, butter and starter cultures
Lactobacillus delbrueckii subsp. *bulgaricus, L. lactis, L. helveticus*	Acid and flavour	Bulgarian butter milk, yoghurt, kefir, kumiss, Swiss, Emmental and Italian cheese
L. acidophilus	Acid	Acidophilus butter milk
Propionibacterium shermanii	Flavour and eye formation	Emmental and Swiss cheese

Generally, three different types of starter cultures are used in dairy industry for the manufacture of a variety of fermented products:

1. Single strain starters

2. Mixed strain starters

3. Multiple strain starters

By and large, all these types are mesophilic (20-30°C) in their growth requirements.

7.3.1 Single Strain Starters

A single strain starter is a pure culture of lactic acid bacteria such as *Lactococcus lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris* etc. This type of culture has the advantage that, if found satisfactory in vigour and flavour, it can give a steady acid production and thereby, a predictable quality of fermented dairy product. This aspect is of considerable economic importance especially in composite dairy plants where a large number of vats are run in series at 10-15 min intervals. However, there is a serious disadvantage with this type of starter as during its application, if it gets attacked by a phage or fails due to any other reason, the quality of the fermented product can be adversely affected.

7.3.2 Mixed Strain Starters

These consist of two or more strains or species and thus, may be more variable in behaviour. The mixed strain starters are generally combinations of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* and the gas and aroma producing mesophilic lactic acid bacteria (*L. lactis* subsp. *diacetylactis* and *Leuconostoc* spp.). Mixed starters are considered safe because if one strain is attacked by a phage, the others usually continue to work because of high phage specificity. A wider tolerance to other factors like temperature and pH changes etc. may be an additional advantage. It has been claimed that mixed starters usually give a single strain on repeated propagation. In other words, mixed starters are difficult to maintain since one strain usually becomes dominant after a few transfers. This is chiefly attributed to the production by the dominant strains of substances such as bacteriocins inhibitory to other strains. Nevertheless, the repressed strains may survive in sufficient numbers to become an

effective starter if the predominant strain is attacked by the phage. In view of this difficulty, if mixed strains are used, each strain should be cultured separately and then mixed together, immediately before milk inoculation.

7.3.3 Multiple Strain Starters

Multiple strain cultures are mixtures of known compatible, non-phage related, carefully selected strains which give generally consistent products when used commercially. Although, their overall phage relationships may be known, the number of individual phage relationships among the strains in these cultures is relatively unknown. A multiple strain starter culture consists of known number of single strains, so that the starter can be used for an extended period of time. These types of starter cultures are being extensively used in New Zealand and other European countries.

7.3.4 Thermophilic Lactic Acid Bacteria (LAB)

Thermophilic LAB (37-45°C) are also used in dairy industry for the manufacture of some fermented products like yoghurt, acidophilus milk and high temperature scalded cheese (Swiss cheese). The examples of thermophilic LAB are S. *thermophilus* and the *Lactobacillus* species. These starters produce lactic acid rapidly at high temperature and the rate of acid production is further enhanced if symbiotic relationships exist between different species, as is the case of yoghurt cultures, namely, S. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Similarly, the combined activity of mesophilic and thermophilic LAB and yeasts leads to lactic acid/alcohol fermentation in milk during the manufacture of Kefir and Kumiss.

7.4 FERMENTATIVE CHANGES IN MILK BROUGHT ABOUT BY STARTER CULTURES

Starter cultures can bring about at least four major fermentation reactions in milk:

1. Lactic acid fermentation

2. Propionic acid fermentation

3. Alcoholic fermentation

4. Citric acid fermentation.

7.4.1 Lactic Acid Fermentation

This is perhaps the simplest type of fermentation—a one step reaction catalyzed by NAD-linked lactic dehydrogenase (pyruvate reductase) which reduces pyruvate to lactate. No gas is formed here. Since, two ATP molecules are consumed in the formation of hexose diphosphate from glucose and four molecules are subsequently produced, the net yield is two ATPs per hexose molecule metabolized. As two hexose molecules are generated from one molecule of lactose, the main milk sugar, there is a net yield of four ATP molecules. This type of fermentation is the first stage in cheese manufacture identical with glycolytic pathway. The lactic fermentation is of two types: homolactic and heterolactic. In homolactic fermentation, lactate or lactic acid is the end product. This fermentation is characteristic of several lactic acid bacteria including *Lactobacillus casei*, *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. However, in heterolactic fermentation, only a half of each glucose molecule is converted into lactate, the other half being converted into other products. Both

these fermentations are responsible for souring or acidification of milk. These fermentations also provide desirable flavours in the fermented product. The lactic acid formation from lactose is shown in the following equation;

$$C_{12}H_{22}O_{11} \quad 2C_6H_{12}O_6 \quad 2CH_3CHOH\ COOH$$
Lactose $\longrightarrow$ Glucose $\longrightarrow$ Lactic acid
(Homolactic fermentation)

Lactose $\longrightarrow$ Glucose $\longrightarrow$ Lactic acid + acetyl methyl
carbinol + diacetoin + diacetyl +
acetic acid
(Heterolactic fermentation)
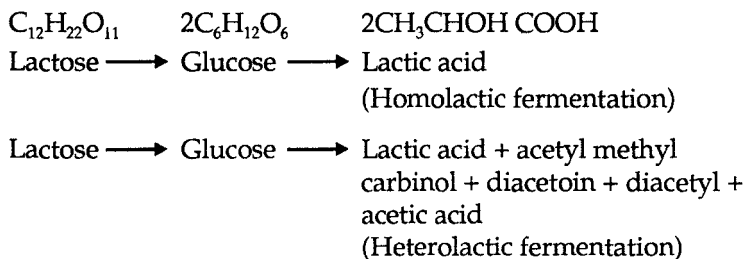
7.4.2 Propionic Acid Fermentation

In this type of fermentation, pyruvate obtained from lactate is carboxylated to yield oxaloacetate which is reduced to succinate and is then decarboxylated to yield propionate. This fermentation is characteristic of *Propionibacterium shermanii* and the fermentation reaction brought about by this organism is summarized below:

$$-6\ (H)$$
$$C_6H_{12}O_6 \longrightarrow 3CH_3CHOH\ COOH \longrightarrow 3CH_3CO\ COOH—$$
Glucose $\qquad$ Lactate $\qquad$ Pyruvate

$$+6\ (H)$$
$$\longrightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$$
$\qquad$ Propionate $\qquad$ Acetate
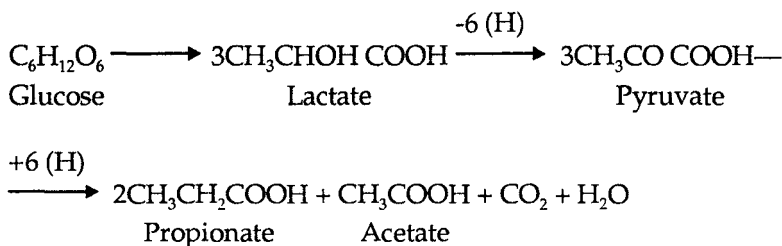
In Swiss cheese, the late formation of CO_2 by the propionic acid fermentation after the completion of lactic fermentation by other starters, is responsible for the formation of holes/eyes while propionic acid contributes to the flavour development.

7.4.3 Alcoholic Fermentation

In this type of fermentation, pyruvate is converted to CO_2 plus acetaldehyde which is then reduced to ethanol in an NAD-linked reaction as shown below:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5\ OH + CO_2$$
Glucose $\qquad$ Ethyl alcohol
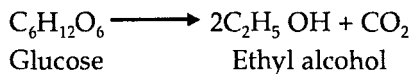
This fermentation is characteristic of yeasts and is uncommon in bacteria. This type of fermentation is typical of Kefir and Kumiss.

7.4.4 Citric Acid Fermentation

Citric acid fermentation is characteristic of *Leuconostoc* species as well as *Lactococcus lactis* subsp. *diacetylactis* which have the ability to metabolize citrate into diacetyl, acetoin and 2,3 -butylene glycol, the major flavouring components in some of the fermented milks like butter, cultured butter milk and cream. The citric acid fermentation reactions are given below:

$$CH_2COOH \cdot HOC \cdot COOH \, CH_2COOH \longrightarrow 2\,CH_3CO\,COOH$$

Citric acid   Pyruvic acid

$$CH_3COHOH \, CH_3 + 2CO_2$$

AMC

$-2H$ $+2H$

$$CH_3CO\,COCH_3$$ $$CH_3CHOH\,CHOH\,CH_3$$
Diacetyl   2,3 - butylene glycol
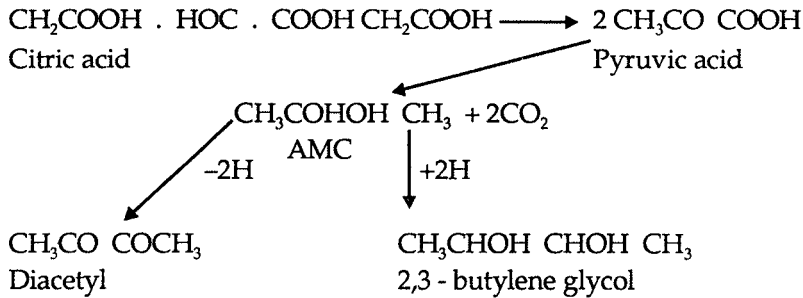
7.5 CLASSIFICATION OF STARTER CULTURES

The starter cultures used in manufacture of cheese and fermented milks belong to several genera of varied physiological properties. (Figure 7.1), which can be classified in different groups using several criteria (Table 7.2). Basically starters can be grouped as lactic or non-lactic starters.

The lactic starters are in maximum use and are generally regarded as lactic acid bacteria. They are Gram positve, non-sporulating, cocci or rods, dividing in one plane (except *Pediococci*), catalase negative, usually non-motile, obligate fermenters mainly producing lactic acid and sometimes volatile acids and carbon dioxide also. The genera included in lactic acid bacteria are *Lactobacillus*, *Lactococcus* (Group N Streptococci), *Streptococcus*, *Leuconostoc* and *Pediococcus*. The classification schemes with differentiating characters are shown in figure 7.2 and 7.3.

Starter Cultures

Lactic   Non-lactic

Rods	Cocci	Bacteria	Molds	Yeasts
Lactobacillus	Lactococcus	*Bifidobacter* spp.	*Penicillium*	*Candida kefir*
	Streptococcus	*Bre. linens*	*camembertii*	*Kluyveromyces*
	Leuconostocs	*Acetobacter acetii*	*P. roquefortii*	*marxianus*
	Pediococcus	*Propionibacterium -*	*Mucor rasmusen*	*Torulospora*
		freudenreichii	*Asp. oryzae*	*delbrueckii*
				Saccharomyces
				cerevisiae

Fig. 7.1 : Starter cultures used in dairy industry.

Table 7.2 : Types of starter cultures

Criteria for grouping	Types of starter	Definition/Explanation
A. Composition of microflora	1. Single strain	Contains only one type/strain of organism.
	2. Mixed strain	Contains a mixture of several strains of lactic organisms in unknown proportion.
	3. Paired compitable strain	Two single strains are used in desired ratio to have better performance.
	4. Multiple strain	Contains a mixture of Known numbers of single strains of desired type.
B. Growth temperature	1. Mesophilic starters	Have optimum temperature for growth between 20-30°C
	2. Thermophilic starters	Have optimum temperature for growth between 37-45°C
C. Production of end products	1. Lactic	Produces lactic acid as the principal end product
	2. Non-lactic	Produces other end products. Lactic acid may be one of the end products.
D. Type of fermentation	1. Homo-fermentative	Produces mainly lactic acid
	2. Hetero-fermentative	Produces lactic acid. CO_2, acetate, ethanol etc.
E. Physical form	1. Liquid	Cultures available in fluid state
	2. Frozen	Cultures available in frozen state
	3. Frozen concentrated	Cultures arc concentrated to have high number of cells and then frozen
	4. Dried cultures (powder, granules)	Culture dehydrated by freeze drying or other techniques to have higher shelf life.
F. Product for which used	The cultures are named after product for which they are meant e.g. Yoghurt culture, dahi culture, kefir culture, cheese culture, etc.	

	Homo Fermentative				Hetero Fermentative
Fermentation of Glucose					
	Lactococcus	Streptococcus	Enterococcus	Pediococcus	Leuconostocs
1. Growth at 10°C	+	–	+	+	+
2. Growth at 45°C	–	+	+	+	–
3. Examples of species used	*Lc. Lactics subsp. lactics, cremoris or diacetylactis*	*S. thermo-philus*	*E. facecium E. liquefaciens*	*P. acidilactici P. pentosaceus*	*Leuconostoc mesenteroides subsp. Cremoris or dextranicum*

Fig. 7.2 : Classification of spherical shaped lactic starters based on fermentation of glucose.

Fermentation of Glucose

Characteristics	Group I (Thermobacterium) Obligately Homofermentative	Group II (Streptobacterium) Facultatively heterofermentative	Group III (Betabacterium) Obligately Heterofermentative
1. Growth at 15°C	–	+	+
2. Growth at 45°C	+	–	+
3. Fermentation of ribose	–	+	+
4. Gas production from gluconate	–	+	+
5. Example of species used	*Lb. delbrueckii subsp. lactis and bulgaricus Lb. acidophilus Lb. helveticus*	*Lb. casei Lb. plantarum*	*Lb. kefir Lb. fermentum Lb. brevis*

Fig. 7.3 : Classification scheme for lactobacilli.

7.6 DESIRABLE CHARACTERISTICS OF A GOOD STARTER CULTURE

While selecting a particular lactic starter culture, either single or in combination with others, for the preparation of fermented dairy products, the following desirable characteristics must be looked into for exploring their full fermentation potentials.

(1)   The lactic starter culture must produce sufficient lactic acid at such a rate that the fermentation process continues smoothly as per the plant schedule.

(2)   A good lactic starter culture must continue acid production over the entire range of temperatures, at which it is likely to be used during the processing, e.g., 38.9 to 40°C in case of Cheddar cheese and 45.6°C and above for Swiss cheese and Brick cheese. Since *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* do not produce lactic acid at such high temperatures, other cultures must be used under these conditions.

(3)   Lactic starters should be resistant to antibiotics and bacteriophages. At the same time, these should be as active as possible in the presence of residual amounts of chemicals/sanitizers/detergents or other inhibitory substances in milk.

(4)   A good starter culture should not produce a bacteriocin (proteinaceous substance inhibitory to related strains) or bacteriocin-like substances or any other antibiotic type of substance inhibiting other strains in the mixed culture.

(5)   Starter culture must not produce undesirable body characteristics, flavour and aroma. In this context, the use of cultures producing ropy body, malty flavour and similar defects should be immediately discontinued.

(6)   In case of mixed cultures (e.g. a combination of *L. lactis* subsp. *lactis* and *Leuconostoc* spp. used for acid and flavour production), the individual cultures must be able to synergize maximum aroma and flavour production without inhibition of acid production. This associative action must be quite stable and contributes towards the development of a good starter culture even after repeated sub-cultures.

(7)   Pure, free from contaminants.

(8)   Stable during sub-culturing.

(9)   Controlled proteolytic and lipolytic activities.

(10)   Compatible with other desirable cultures (symbiotic)

(11)   Tolerant to pH, salt etc. when required.

(12)   Tolerant to processing changes.

(13)   Improve the nutritive value of the fermented product.

(14)   Should be able to inhibit undesirable types of organisms and pathogens.

(15)   Its maintenance should be easy.

(16)   Special dietic cultures should be resistant to adverse conditions in the alimentary tract.

7.7 EVALUATING THE QUALITY OF STARTER CULTURES

For judging the starter culture for routine use, following types of examinations are done.

Activity Test—This test is a measure to check the efficiency of starter culture to bring about a most desirable change in milk by its metabolic activity. Generally, it is measured as a rate of acid production under a specified set of conditions. This helps in fixing the time schedule and get predictable quality product.

The procedures of activity test may vary depending on the starter, the product and the manufacturing conditions. Based on activity test, the cultures are also designated as 'fast' or 'slow'.

The activity test for yoghurt culture is outlined as follows, as an example.

Reconstituted Skim Milk (10-12% TS)

↓

Autoclave

↓

Inoculate Yoghurt Cultuie @ 2%

↓

Mix

↓

Incubate at 42°C for 3 hrs.

↓

Measure titratable acidity

↓

0.8-0.9% Lactic acid is considered satisfactory

↓

Yoghurt Culture
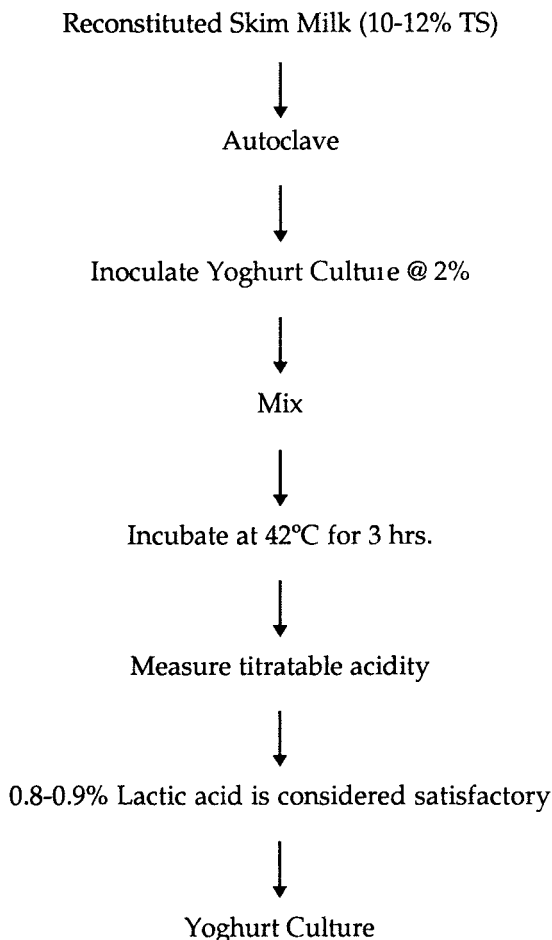
Chemical Tests—Chemical tests include—(i) titratable acidity, (ii) volatile acidity, (iii) diacetyl estimation, (iv) acetaldehyde estimation

Microbiological Tests—These tests are done to check the purity and viability of the culture. These tests include—(1) Gram staining, (2) Catalase test, (3) Total viable count, (4) MPN for coliforms, (5) Tests for yeasts and molds, (6) Proteolytic activity, (7) Lipolytic activity, (8) Phage detection test, etc.

Bacteriophage is one of the important causes for starter failure. Several methods are available for its detection in dairy industry, but one of them is presented in Fig. 7.4.

Swab Tests—Swab tests on starter culture equipment and propagation vessels are essential to check on the efficiency of cleaning, and the sanitary condition of the plant.

Take 100 ml of pasteurized
vat milk or autoclaved milk
↓
Add 1 ml of guaranteed active
working culture
↓
Mix
↓
Distribute in 10 ml aliquotes
in test tubes
↓
Keep one tube as control
(without adding anything)
add in remaining tubes
add 1 ml of appropriately diluted
samples of bulk culture,
whey taken from different vats at
different stages, rinsings, etc.
to be tested for phage
↓
Close tubes with sterile rubber bungs
↓
Mix
↓
Incubate at 30°C for 6 h
↓
Measure titratable acidity.
If acidity in any tube is 10-15% less
than control tube, then the phage
infection is established

Fig. 7.4 : Method for detection of bacteriophage.

7.8 PROPAGATION OF STARTERS

Large quantities of starter culture in active and pure form is essential to the success of starter in product manufacture. This can be achieved by careful propagation of cultures.

The propagation is required to maintain activity of the culture and also to increase the number of cells and volume of the culture for inoculation into the milk for product manufacture. Strict asepsis is required to maintain the purity of starters during propagation. Similarly, the medium (milk) should be free from antibiotic residues or any other substances harmful to the starter. Control of temperatures during incubation and cooling are also important factors affecting the activity of culture.

A typical flow diagram for propagation of starters is shown as follows:

Milk

$\downarrow$

Heat treatment (sterilization/80-90°C for 10-30 min)

$\downarrow$

Cooling upto optimum temperature for the culture

$\downarrow$

Inoculation with active culture @ 0.5 to 2.0%

$\downarrow$

Incubation at optimum temperature of the culture

$\downarrow$

Coagulation

$\downarrow$

Cooling at < 5°C
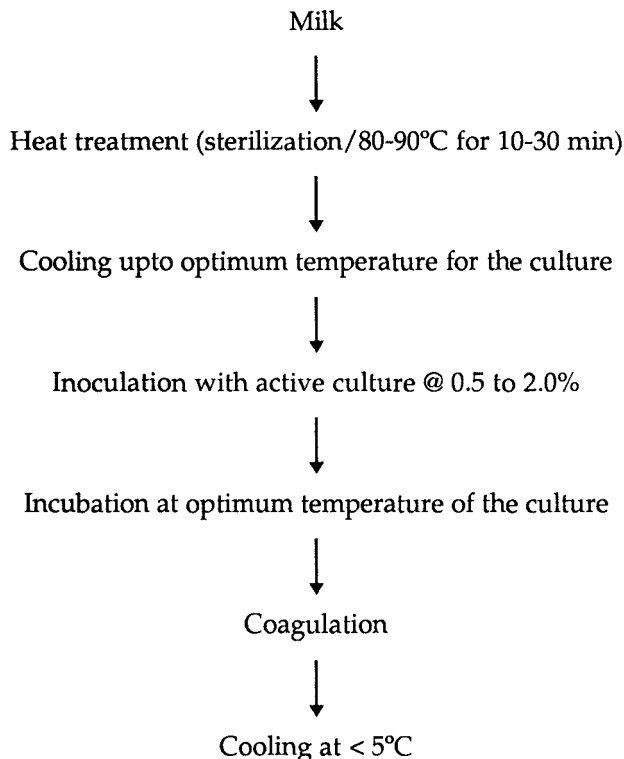
7.9 MAINTENANCE AND PRESERVATION OF STARTER CULTURES

A variety of methods can be used for the preservation and maintenance of starter cultures. Proper preservation of starter is extremely important step in the manufacture of fermented dairy products since many times the mother culture or bulk cultures get inactivated. In the event of sudden starter failures, the availability of suitably preserved starters can come to the rescue of dairy technologists. The methods of preservation of starter cultures in vogue all over the world are listed below.

1. Liquid Starter
2. Dried Starter
    (i)   Spray Dried
    (ii) Freeze Dried or Lyophilized
    (iii) Concentrated Freeze Dried
3. Frozen Starter
    (i)   Deep Frozen at -40°C
    (ii) Ultra low temperature freezing at -196°C under liquid nitrogen
4. Starter Concentrates for direct vat inoculation

7.10 STARTER CULTURE PROBLEMS

The use of starter cultures in the production of dairy products is not without problems. These can arise during the production of the starter and during its use, resulting

in **Slow acid production,** when lactic acid is generated at a much slower rate than normally expected or in extreme cases not at all. Slow acid production by a starter can lead to complete product failure or a poor quality product showing increased incidence of spoilage or even pathogen problems, e.g. the growth of enterotoxigenic strains of *Staphylococcus aureus* or survival of *Salmonella* spp.

The main reasons for starter culture problems are:

(1) Bacteriophages—Bacteriophages originating from raw milk can attack starter culture organisms during starter production and use. This is by far the most important cause of starter culture failure and is of particular importance in large scale semi-continuous cheese manufacture in which bacteriophage activity can have very serious effects on production.

(2) Antibiotic residues in milk—If antibiotics are present in the milk used for starter production or as a raw material, they can kill or inhibit starter culture organisms or destroy the balance between organisms in a mixed starter. Antibiotics can be introduced into milk when udder injections of antibiotic are used to treat mastitis and recommended withholding times are not adhered to. The problem has largely been solved by the use of routine quality control checks on milk for the presence of antibiotics and financial penalties imposed on farmers producing milk containing antibiotics.

S. thermophilus is found to be most sensitive to penicillin residues in milk.

(3) Inadequate rinsing of equipment—If equipment is not rinsed adequately after the use of sanitizers, starter quality can be inhibited by the pressure of sanitizer residues in the milk. QUATs are particularly important.

(4) Presence of agglutinins—The presence of naturally occurring agglutinins in milk (antibodies that cause bacterial cells to clump together) can cause starter culture organisms to produce clumps, resulting in slow acid production.

(5) Instability of functional plasmids—Lactic acid bacteria contain plasmids that are responsible for determining a number of important starter properties, i.e. lactose metabolism, proteinase production, bacteriophage resistance and production of polymers. Plasmids can be lost during the subculturing stages employed in starter production, resulting in slow acid production or other defects.

7.11 CAUSES AND REMEDIES FOR SLOWNESS OF STARTER CULTURES

Starter cultures may show a number of defects with respect to growth and performance. Among these, the defects called weak acid, no coagulation, flat flavour, thin body, etc. are due to slowness or sluggishness of the starter. This gives inadequate performance during product manufacture leading to delay in manufacture, poor quality product and economic loss.

Causes and remedies for slowness of starter cultures are summarized in Table 7.3.

Table 7.3 : Causes and remedies for slowness of starter cultures

Causes	Remedies
A. **Problem with starter itself**	
1. Spontaneous loss of vitality	Replace culture
2. Strain variation	Check balance

... contd.

Causes	Remedies
3. Physiological state of cells	Inoculate at correct stage
B. Incompetence in the control of starter	
1. Starter contaminated	Prevent contamination
2. Too infrequent subculturing	Subculture regularly at right stage
3. Use of unsuitable media (milk)	Use high quality reconstituted skim milk for propagation
4. Culturing at wrong temperature	Ensure proper temperature
C. Problem with milk	
1. Abnormal milk e.g. Mastitic, Colostrum, Late lactation	Don't allow mixing with normal milk
2. Antibiotic residues in milk	Don't mix milk from antibiotic treated animal for 3 days
3. Milk affected by feeds, seasonal factors, aeration, etc.	Don't use such milk or use after proper treatment
4. Inhibitory substances in milk, e.g. preservative, detergent & sanitizer residues	Avoid such milk
D. Problem with production method	
1. Changes in ripening time and temperatures (in cream ripening for butter manufacture)	Ensure proper adjustments of time and temperatures
2. Cooking and clotting temperature (in cheese making)	Use resistant cultures, Don't change temperatures
E. Bacteriophage action	Observe strict asepsis, use starter rotation system, use PRM/PIM, environmental control, use of modified cultures.

7.12 PRODUCTION OF BACTERIOCINS

Lactic acid bacteria are exerting antagonistic effect against several other organisms, due to production of several antimicrobial substances. These include lactic acid, acetic acid, other organic acids, hydrogen peroxide, diacetyl, reduced pH and Eh and a number of bacteriocins.

Bacteriocins are the proteins that are inhibitory to self or closely related species. However, some of the bacteriocins of lactic acid bacteria have shown wide spectrum activities (Table 7.4). The exact mechanisms for synthesis and other characteristics of many bacteriocins are still not clear. However, the nisin is the only one which is fully characterised and used as food preservative.

Table 7.4 : Bacteriocins of lactic acid bacteria

Bacteirocin	Producer	Inhibitory spectrum
Fermenticin	*Lb. fermenti*	Lactobacilli
Brevicin	*Lb. brevis*	Pediococci, Lactobacilli. Leuconostocs, *N. coralina*
Helveticin J	*Lb. helveticus*	*Lb. bulgaricus. Lb. lactis, Lb. helveticus*
Lactacin B	*Lb. acidophilus*	Lactobacilli
Lactocidin	*Lb. acidophilus*	Broad antibiotic spectrum, Gram +ve & Gram -ve Bacteria
Acidophilin	*Lb. acidophilus*	Lactic acid bacteria, Spore formers, *Salmonella* spp., *E. coli, S. aureus*, Pseudomonas
Acidolin	*Lb. acidophilus*	Broad antibiotic spectrum, Spore formers, Enteric pathogens
Reuterin	*Lb. reuteri*	Salmonella, Shigella, Clostridia, Staphylococci, Listeria. Candida, Trypanosoma
Nisin	*Lc. lactis*	Lactococci, Bacilli, Clostridia, Micrococci, *S. aureus*
Diplococcin	*Lc. lactis* ssp *cremoris*	*Lc. lactis* spp *lactis. Lc. lactis* ssp *cremoris*
Lactococcin	*Lc. lactis* ssp *lactis*	Lactococci, *S. aureus, B. cereus, S. typhi*
Mesenerocin	*Leu. mesenteroides*	*L. monocytogens, B. linens, E. faecalis, P. pantosaceus*
Carnocin	*Leu. carnosum*	Lactobacilli, Pediococci, Enterococci, Leuconostocs, Carnobacteria, Listeria
Pediocin AcH	*P. acidilactici*	Lactobacilli, *S. aureus,* Leuconostocs, *P putida, L. monocytogens, Cl. perfringens*

□□□

8

METABOLISM OF STARTER CULTURES

8.1 INTRODUCTION

Louis Pasteur, in 1857, was the first to demonstrate that the lactic fermentation was of microbial origin and proposed "Germ Theory of Fermentation". In 1887, Lister isolated pure cultures of the lactic acid bacteria responsible for milk acidification. In 1880s, Conn. in U.S.A., Storch in Denmark, and Weigmann in Germany demonstrated the advantages of starter cultures in dairy industry.

In dairy fermentations, starter cultures include lactic acid bacteria, propioni bacteria, surface ripening bacteria, yeasts and molds. Lactic acid bacteria are responsible for the acidification that occurs during the manufacture of all fermented dairy products. Because of the central role that these organisms play, this chapter primarily focuses on their metabolism.

When starter culture grows in milk, it affects the constituents of milk and brings fermentative metabolic changes. It will produce different intermediary or end products, which give typical attributes to fermented milk.

8.2 CARBOHYDRATE METABOLISM—GENERAL

Lactic acid bacteria, as non-respiring organisms, principally generate adenosine triphosphate (ATP) by fermentation of carbohydrates coupled to substrate-level phosphorylation. The two major pathways for metabolism of hexoses (i.e., glucose) in these organisms are the homofermentative (Embden-Meyerhof) and heterofermentative (phosphoketolase) pathways. The homofermentative pathway (Fig 8.1) theoretically yields 2 moles of lactate and 2 ATPs per hexose. This pathway used by all lactic acid bacteria except *Leuconostoc* spp. and hetero-fermentative lactobacilli. These organisms use the heterofermentative pathway (Fig. 8.2), which theoretically yields 1 ATP and 1 mole each of lactate, ethanol, and CO_2 per hexose. Differences in metabolic end products have a

significant impact on the final product. For example, production of CO_2 can result in formation of holes (eyes) within the cheese and, depending on which cheese variety is being produced, this can be either a quality attribute or a defect. Additionally, production of ethanol, via esterification with short-chain fatty acids, can result in fruity flavour defects in cheddar cheeses. For more detailed descriptions of carbohydrate fermentation and energy transduction in lactic acid bacteria, see Axelsson (1993), Monnet *et al.* (1996), and Poolman (1993).

GLUCOSE
ATP
ADP

Glucose-6-P

Fructose-6-P
ATP
ADP

Fructose-1,6-DP

Glyceraldehyde-3-P ⇌ Dihydroxy-acetone-P
2Pi
2 NAD$^+$
2 NADH+2H$^+$

2 1,3-diphosphoglycerate
2 ADP
2 ATP

2 3-phosphoglycerate

2 2-phosphoglycerate
H_2O

2 phosphenolpyruvate
2 ADP
2 ATP

2 pyruvate
2 NADH+2H$^+$
2 NAD$^+$

2 LACTATE

Fig. 8.1 : The homofermentative (Embden-Meyerhof) pathway.

GLUCOSE
ATP
ADP

Glucose-6-P
NAD$^+$
NADH+H$^+$

6-phospho-gluconate
NAD$^+$
CO$_2$
NADH+H$^+$

Ribulose-5-phosphate

Xylulose-5-phosphate
Pi

Glyceraldehyde-3-P
Pi
NAD$^+$
NADH+H$^+$

1,3-diphosphoglycerate
ATP
ADP

3-phosphoglycerate

2-phosphoglycerate
H$_2$O

phosphenolpyruvate
ADP
ATP

pyruvate
NADH+H$^+$
NAD$^+$

LACTATE

Acetyl-phosphate
CoA
Pi

Acetyl-CoA
NADH+H$^+$
CoA
NAD$^+$

Acetaldhyde
NADH+H$^+$
NAD$^+$

ETHANOL

Fig. 8.2 : The heterofermentative (phosphoketolase) pathway.

8.3 LACTOSE METABOLISM

Lactose, a disaccharide composed of glucose and galactose, is the principal carbohydrate present in milk (45-50 g/L). Therefore, lactose metabolism is primarily responsible for production of energy by lactic acid bacteria growing in milk. The pathway used to metabolize lactose differs among lactic acid bacteria. These pathways can be divided into two groups based on whether the lactose is transported by a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) or a lactose permease.

8.3.1 Organisms that Transport Lactose via a PEP-Dependent PTS

Lactococcus lactis and *Lb. casei* translocate lactose via a PEP-dependent PTS. In these organisms, lactose enters the cytoplasm as lactose-phosphate and is hydrolyzed by phospho-β-galactosidase to yield galactose-6-phosphate and glucose. Galactose-6-phosphate is metabolized by the tagatose-6-phosphate pathway (Fig. 8.3) and the glucose moiety is metabolized by the Embden-Meyerhof pathway (see Fig. 8.1). In both pathways, an aldolase cleaves a diphosphate intermediate compound to produce the triose phosphate—dihydroxyacetone phosphate and glyceraldehyde phosphate. These triose pathways are converted to pyruvate at the expense of NAD^+. Regeneration of NAD^+ is usually accomplished by reducing pyruvate to lactate. Two enzymes, L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), are responsible for conversion of pyruvate to L-lactate and D-lactate, respectively. Lactococci produce only L-lactate whereas *Lb. casei* forms either only L-lactate or both L- and D-lactate, depending on the subspecies.

Galactose-6-P

Tagatose-6-P

ATP

ADP

Tagatose-1,6-DP

Dihydroxy-⇌ Glyceraldehyde-3-P
acetone-P

Embden-Meyerhof Pathway
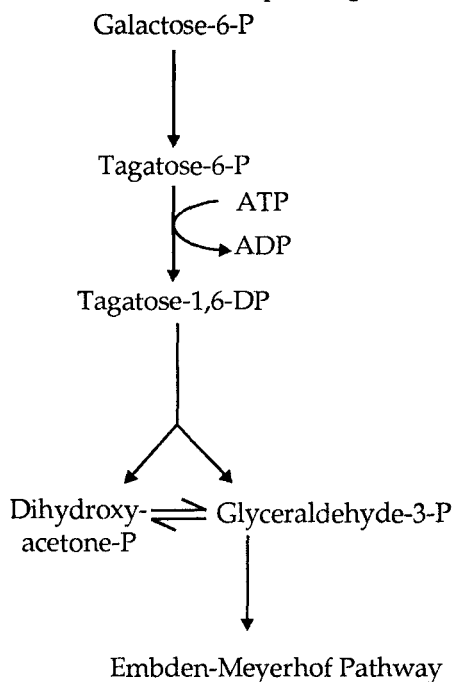
Fig. 8.3 : The tagatose-6-phosphate pathway.

The efflux of lactate ions and protons via a symport mechanism provides a secondary mechanism by which energy can be generated. This efflux results in formation of a proton gradient across the membrane which can be used for transport of nutrients or to synthesize ATP via a reversible ATPase present in the cytoplasmic membrane.

To ensure the availability of PEP for use in translocating lactose and other carbohydrates into the cytoplasm, the intracellular PEP pool (PEP, 2-phosphoglycerate, and 3-phosphoglycerate) is regulated. This is accomplished by regulation of pyruvate kinase activity, the enzyme that catalyzes the conversion of PEP to pyruvate, by inorganic phosphate (P_i) and fructose, 1.6 diphosphate (FDP), an intermediate compound in the Embden-Meyerhof

pathway. These compounds allosterically regulate pyruvate kinase, with FDP being an activator and P_i being an inhibitor. When cells are rapidly fermenting lactose, the intracellular concentration of level of FDP is high and P_i level is low, resulting in an active pyruvate kinase and formation of pyruvate. However, when lactose becomes limiting, the level of FDP is greatly reduced and P_i increases resulting in inhibition of pyruvate kinase and accumulation of a PEP pool.

In the 1930s, the observation was made that the ability of lactococcal strains to metabolize lactose was an unstable trait. In the late 1970s, McKay and coworkers were able to demonstrate that the high rate of spontaneous loss of the ability to ferment lactose by lactococci resulted from the loss of plasmid DNA. Similar results have demonstrated that the ability to metabolize lactose is also plasmid encoded in strains of *Lb. casei*. Subsequently, a DNA fragment coding for lactose metabolism was cloned from lactococci and its nucleotide sequence determined.

Analysis of this nucleotide sequence revealed that the fragment encoded for lactose-specific enzymes of the PTS, enzymes of the tagatose-6-phosphate pathway, phospho-β-galactosidase, and a repressor. The genes encoding the enzymes involved in lactose metabolism are organized in an operon, and the repressor (LacR) is divergently transcribed. Binding of LacR to operators in the inter-cistronic region between the *lac* operon and *lacR* activates transcription of *lacR* and represses transcription of the *lac* operon. Binding of tagatose-6-phosphate by *lacR* results in release of *lacR* from the operator and consequently the lac operon is transcribed. Transcription of the *lac* operon is also decreased by presence of glucose. This is most likely caused by lower intracellular levels of cyclic adenosine monophosphate (AMP) resulting in catabolite repression, as has been described in numerous other microorganisms. Regulation at the level of transcription allows the organism to avoid synthesizing enzymes required for lactose metabolism when lactose is not present, or when a more readily used carbohydrate such as glucose is present. Additionally, this mechanism of regulation allows the organism to rapidly produce these enzymes in case lactose becomes available.

8.3.2 Organisms that Transport Lactose via a Permease

Lactobacillus delbrueckii, Lactobacillus helveticus, Leuconostoc spp., and *S. thermophilus* transport lactose without modification via a lactose permease. There are two distinct types of lactose permeases. *Lb. helveticus* and *Leuconostoc* spp. are believed to transport lactose in symport with a proton. *S. thermophilus* and *Lb. delbrueckii* transport lactose via a lactose-galactose antiport system. Regardless of the type of permease, the intracellular lactose is hydrolyzed by β-galactosidase to yield glucose and galactose. The glucose moiety is metabolized via the Embden-Meyerhof pathway (see Fig. 8.1) by *S. thermophilus, Lb. delbrueckii,* and *Lb. helveticus. Lb. helveticus* metabolizes the galactose moiety via the Leloir pathway (Fig. 8.4). Regeneration of NAD^+ typically is accomplished via an LDH. *S. thermophilus, Lb. delbrueckii,* and *Lb. helveticus* produce L-lactate, D-lactate, and both L- and D-lactate, respectively.

Leuconostoc spp. convert galactose to glucose-6-phosphate via the Leloir pathway (see Fig. 8.4), which then enters the heterofermentative pathway (see Fig 8.2). The glucose moiety also is metabolized by the heterofermentative path-way. *Leuconostoc* spp. express only D-LDH and hence only D-lactate is formed.

Galactose

|⟋ ATP

⟍→ADP

↓

Galactose-1-P

|

↓

Glucose-1-P

|

↓

Glucose-6-P

|

↓

Embden-Meyerhof Pathway
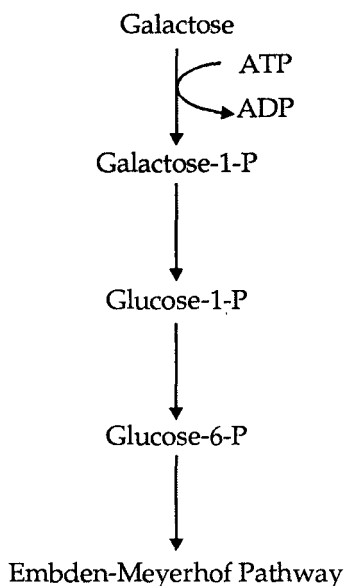
Fig. 8.4 : The Leloir pathway.

8.4 CITRATE METABOLISM

Relatively small quantities (0.15%-0.2%) of citrate are present in milk and the ability to metabolize citrate among dairy starter cultures is limited to *Leuconostoc* spp. and biovariants of *Lc. lactis*, designated Cit$^+$ *Lc. lactis* strains or *Lc. lactis* subsp. *lactis* biovar *diacetylactis* (Monnet *et al.*, 1996). The metabolic end products of citrate metabolism are diacetyl, acetoin, 2,3-butanediol, acetic acid, and carbon dioxide. Diacetyl is an important flavour compound in a variety of fermented milks and sour cream, and is believed to be important in cheddar cheese flavour. Acetoin and 2,3-butanediol, both flavourless compounds, are produced from diacetyl by 2,3-butanediol dehydrogenases (Crow, 1990). The carbon dioxide produced is responsible for the holes (eyes) in Gouda and Edam cheeses and the effervescent quality of buttermilk.

Citrate is transported without modification into the cell by a citrate permease, which is plasmid encoded in lactococci and *Leuconostoc* spp. (Kempler and McKay, 1981; Vaughan et al., 1995). The intracellular citrate is then hydrolyzed by citrate lyase to yield oxaloacetate and acetate; the oxaloacetate is then decarboxylated to pyruvate. Five pathways exist for the metabolism of pyruvate: lactate production via an LDH, formate and acetate or ethanol production via pyruvate-formate lyase, acetate and CO_2 production via pyruvate dehydrogenase, acetate and CO_2 production via' pyruvate oxidase, and the diacetyl/lacetoin pathway (Fig. 8.5). In the diacetyl/acetoin pathway, a pyruvate molecule is decarboxylated to acetaldehyde-thiaminpyrophosphate (TPP). α-Acetolactate synthase catalyses the condensation of acetaldehyde-TPP with another molecule of pyruvate to form α-acetolactate. It is generally recognized that diacetyl is formed by the non-enzymatic decarboxylative oxidation of α-acetolactate. However, some researchers postulate that an enzyme (diacetyl synthase) exists that is capable of forming diacetyl directly from acetaldehyde- TPP.
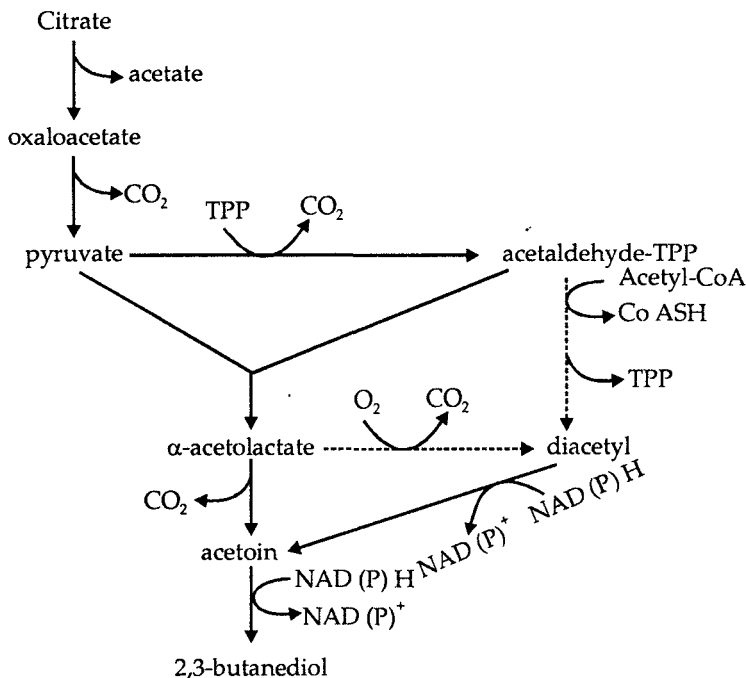
Fig. 8.5 : The diacetyl/acetoin pathway. The line with longer dashes indicates a non-enzymatic reaction (α-acetolactate to diacetyl). The line with shorter dashes indicates that the reaction is hypothetical (acetaldehyde-TPP to diacetyl).

Hugenholtz (1993) described strategies for use of genetic engineering to construct strains of lactococci that produce elevated levels of diacetyl. These include inactivation of genes coding for enzymes involved in the metabolism of pyruvate that do not lead to diacetyl (i.e., LDH), increasing the activity of α-acetolactate synthase and inactivating the gene coding for the enzyme (α-acetolactate decarboxylase), which converts α-acetolactate to acetoin. The intent of these manipulations is to increase the quantity of pyruvate that enters the diacetyl/acetoin pathway and to eliminate branches in the pathway that do not lead to diacetyl.

8.5 PROPIONIC ACID FERMENTATION

Propionibacterium freudenreichii subsp. *shermanii* is a component of the starter culture used to manufacture Swiss-type cheeses. The maturation process of Swiss-type cheeses includes holding the cheese at 7°C for a few days and then elevating the ripening temperature to 20°C to 26°C. This increase in temperature is required to permit growth of *P. freudenreichii* subsp. *shermanii*. This organism metabolizes lactate produced by fermentation of lactose by other components of the starter culture, S. *thermophilus* and *Lb. helveticus*. The metabolism of lactate by propionibacteria results in formation of propionate, acetate, and CO_2, the fermentation is typically described by the following equation (Frank and Marth, 1988):

$$3 \text{ lactate} \rightarrow 2 \text{ propionate} + 1 \text{ acetate} + 1CO_2$$
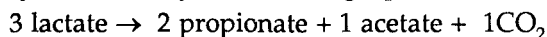
The metabolic pathway used by propionibacteria in the fermentation of lactate is complex and involves two connected cycles (Fig. 8.6), with the organism generating 1 mole of ATP per mole of lactate.
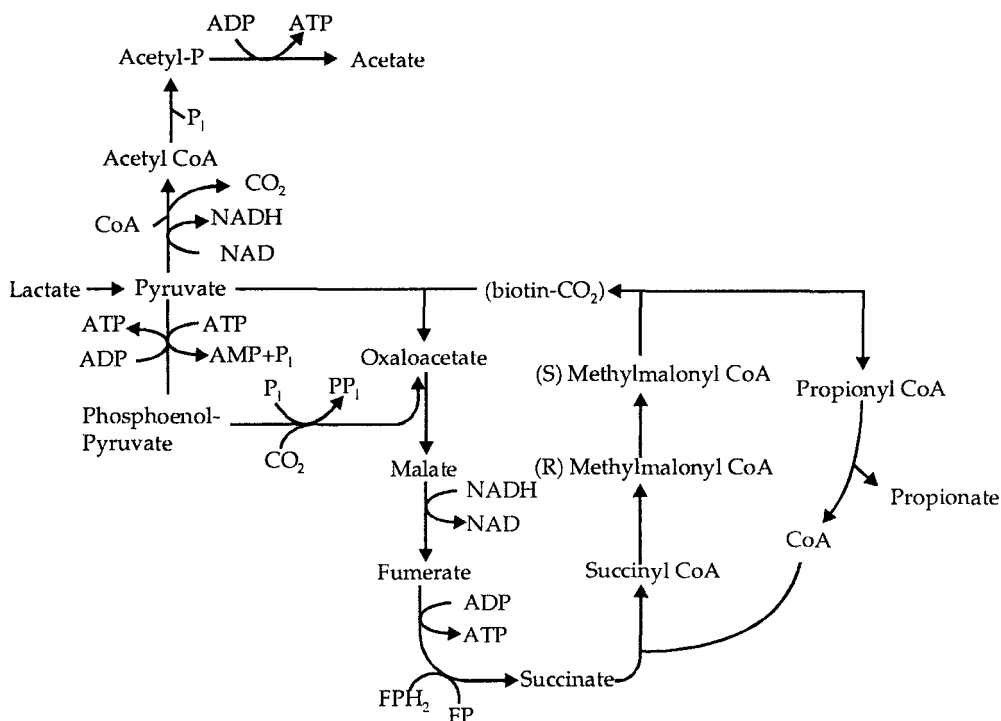
Fig. 8.6 : Metabolic pathway for the fermentation of lactate by propionibacteria.

Fermentation of lactate by propionibacteria in the cheese matrix is more complicated than that described in Figure 8.6 and summarized in the equation given previously. Crow (1986) demonstrated that metabolism of aspartate to succinate by *P freudenreichii* subsp. *shermanii* influenced the fermentation of lactate to propionate, acetate, and CO_2. The generation of reducing equivalents from aspartate metabolism results in an increase in the amount of lactate being fermented to acetate and CO_2 rather than propionate. That aspartate is catabolized in Swiss-type cheeses is supported by the presence of succinate in those cheeses and by the observation that the molar ratio of propionate to acetate is typically significantly lower than 2:1.

8.6 PROTEOLYTIC SYSTEMS OF LACTIC ACID BACTERIA

Lactic acid bacteria used in dairy fermentations are amino acid auxotrophs that typically require several amino acids for growth. The quantities of free amino acids present in milk are not sufficient to support growth of these bacteria to high cell density; therefore, they require a proteolytic system capable of using the peptides present in milk and hydrolyzing milk proteins (α_{s1}-, α_{s2}-, and κ-, and β-caseins) to obtain essential amino acids. Additionally, the activity of proteolytic enzymes derived from lactic acid bacteria is necessary for development of flavour in ripened cheeses. Peptides and amino acids formed by proteolysis may impart flavour directly or serve as flavour precursors in fermented dairy products. The resulting flavours may have either a positive or negative impact on the quality of the finished product.

8.6.1 Proteolytic System of Lactococci

The proteolytic system of lactococci has been characterized in significant detail. Many of the enzymes have been purified and characterized, and numerous genes encoding components of proteolytic systems have been sequenced. The proteolytic systems of other lactic acid bacteria are not as well characterized; however, many individual components have been examined. These components, in most instances, have homologs in the lactococcal system. Therefore, the lactococcal system is believed to serve as a general model for the proteolytic systems of other lactic acid bacteria. For extensive reviews of the proteolytic enzyme systems of lactic acid bacteria, see Kunji *et al.* (1996) or Kok and de Vos (1994).

A model of the lactococcal proteolytic system is presented in Figure 8.7. The system can be separated into three components: extracellular enzymes, transport systems, and intracellular enzymes. While growing in milk, lactic acid bacteria obtain essential amino acids in a variety of ways. They first use non-protein nitrogen sources such as free amino acids and small peptides. Casein, which composes 8% of all proteins in milk, becomes the primary nitrogen source after non-protein nitrogen is depleted.
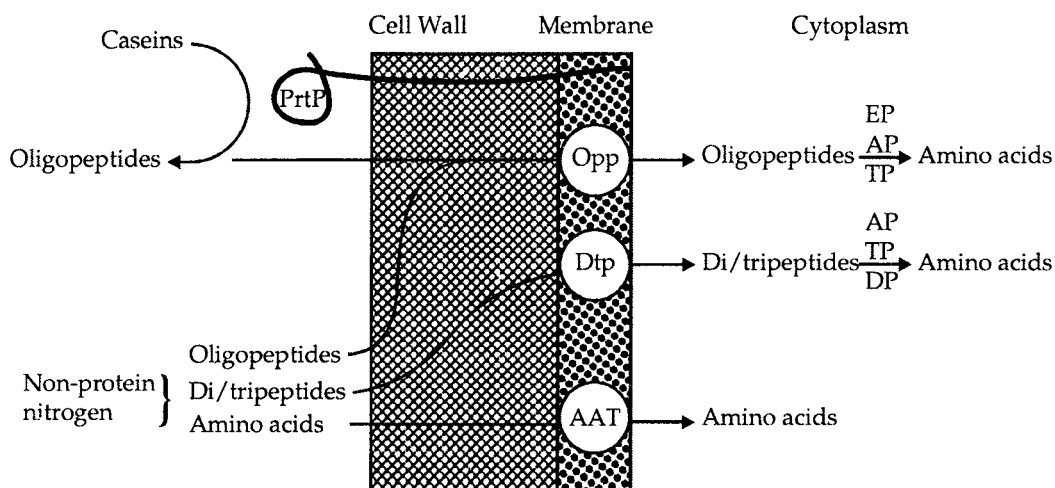
Fig. 8.7 : Schematic representation of the lactococcal proteolytic system. Abbreviations: PrtP, cell-envelope-associated proteinase; Opp, oligopeptide transport system; Dtp, di/tripeptide transport systems; AAT, amino acid transport systems; EP, endopeptidases; AP, aminopeptidases; TP, tripeptidases; DP, dipeptidases.

Extensive investigations have revealed that a cell-envelope-associated proteinase, designated PrtP, is the only extracellular proteolytic enzyme present in lactococci. The enzyme is a serine-protease, which is expressed as a pre-pro-proteinase. A signal peptidase removes the signal peptide upon transport across the cytoplasmic membrane. Subsequently, a lipoprotein maturase (PrtM) is thought to cause a conformational change in the pro-proteinase, resulting in release of the pro-region via autoproteolysis, and an active PrtP. The activated enzyme remains associated with the cell because of the presence of a C-terminal membrane anchor sequence. Genes encoding lactococcal proteinases with different substrate specificities have been sequenced and the amino acid residues involved in substrate binding and catalysis determined. A critical feature of the enzyme is its broad cleavage specificity,

which results in release of more than 100 oligopeptides from soluble-β-casein, 20% of which are small enough to be transported by the oligopeptide transport system (Juillard *et al.*, 1995). Loss of PrtP, which is typically plasmid encoded in lactococcal strains, results in derivatives capable of reaching only approximately 10% of the final cell density of the parental strain, indicating that PrtP is essential for growth of lactococci to high cell density in milk.

8.6.2 Proteolysis and Cheese Flavour Development

Even though flavour development in various types of cheese remains a poorly defined process, it is generally agreed that proteolysis is essential for flavour development in bacterial ripened cheeses (Fox *et al.*, 1993; Visser, 1993). Proteolytic enzymes present in this group of products include chymosin, plasmin, and proteolytic enzymes from starter cultures, adjunct cultures, and non-starter lactic acid bacteria. The specificities and relative activities of the proteolytic enzymes present in the cheese matrix determine which peptide and amino acids accumulate and, hence, how flavour develops. The use of isogenic strains, differing only in the specificity of activity of a single proteolytic enzyme, will have great utility in elucidating the role of individual enzymes in cheese flavour development.

Free amino acids and peptides in the cheese matrix can contribute to cheese flavour either directly or indirectly and with positive or negative effects. Cheese flavour development has been the subject of numerous comprehensive reviews (Fox *et al.*, 1993; Urbach, 1995). A major negative effect of proteolytic products is bitterness, which is believed to be caused by hydrophobic peptides ranging in length from 3 to 27 amino residues (Lemieux and Simard, 1992). They can be generated either from casein directly by chymosin or via the joint action of chymosin and cell-envelope-associated proteinases from lactic acid bacteria; they can be hydrolyzed to nonbitter peptides and amino acids by peptidases of lactic acid bacteria. Therefore, accumulation of bitter peptides is dependent on the relative rates of their formation and hydrolysis. A variety of volatile compounds can be derived from catabolism of amino acids (Hemme *et al.*, 1982). Numerous sulphur-containing compounds, particularly methanethiol, are thought to be important in cheese flavour. Formation of methanethiol by lactococci is believed to result from the action of either cystathionine β-lyase (Alting *et al.*, 1995) or cystathionine γ-lyase (Bruinenberg *et al.*, 1997) on methionine. Alternatively, amino acid catabolism can give rise to compounds that have a negative impact on cheese flavour. For example, catabolism of aromatic amino acids can give rise to compounds such as indole and skatole, which contribute to unclean flavours in cheese. Overall, proteolysis is believed to be essential for development of characteristic flavour compounds in bacterial ripened cheeses; however, the mechanisms by which products of proteolysis give rise to beneficial flavour compounds remains unknown. Additionally, other than bitterness, the mechanisms by which proteolysis impacts on the development of undesirable flavour compounds are unknown.

□□□

9

GENETICS OF STARTER CULTURES

9.1 INTRODUCTION

The production of fermented dairy products, that is, cheese, yoghurt, sour cream, and butter, depends greatly upon the activities of lactic acid bacteria which are used as starter cultures. These organisms are responsible for the hydrolysis of milk proteins, lactose, and citrate and the production of acid and flavour compounds, as well as other characteristics of the end-product.

The genetic instability of starter cultures, bacteriophage infection, production of off-flavours, and insufficient development of acid during fermentation are costly problems for the dairy industry. Therefore there is a great deal of interest in the development of new and improved strains, in particular the construction of strains using the modern tools and techniques of recombinant DNA technology.

Since the early 1970s much research has focused on obtaining fundamental information on the genes, plasmids and gene transfer mechanisms of lactic acid bacteria. This chapter reviews plasmid biology and gene transfer mechanisms of starter cultures, alongwith genetic modification of lactic acid bacteria using recombinant DNA technology.

9.2 PLASMIDS AND PLASMID-LINKED TRAITS IN STARTER CULTURE BACTERIA

Starter culture bacteria, like other bacteria, carry genetic information (genetic code) in the circular chromosomal DNA, circular plasmids, and linear transposons. Chromosomal DNA carries genetic codes for vital functions of a cell (such as a key enzyme in the EMP or HMS pathways in lactic acid bacteria). Although both plasmid DNA and transposons can carry genetic codes, those are only for non-vital functions, i.e., characteristics that are not absolutely necessary for the survival of a cell (such as the ability to hydrolyze a large protein). However, having such a genetic code gives a cell a competitive advantage over the other

cells without it, but sharing the same environment. Initial research in the early 1970s revealed that many industrially important phenotypes in different lactic acid bacteria are plasmid linked. Since then, due to the availability of specific techniques, the genetic basis of many plasmid-encoded important phenotypes in several starter culture bacteria, particularly in *Lactococcus lactis*, have been studied. These studies have not only helped to identify the locations of many genes, their structure, and the control systems involved in their expression, but also enabled the researchers to transfer it into a cell lacking a specific phenotype, and to create a new desirable strain. Characteristics of plasmids and some plasmid-linked traits in starter culture bacteria are discussed here.

9.2.1 Important Characteristics of Bacterial Plasmids

- They are double stranded, circular, self-replicating DNA that can differ in size (<1 to >100 kb).
- May not be present in all species or all strains in a species.
- A strain can have more than one type of plasmid that differs in size and the genetic code it carries.
- A plasmid can he present in more than one copy in a cell (copy number; this is in contrast to single copy of chromosome that a cell can carry).
- For some plasmids, copy numbers can be reduced (depressed) or increased (amplified) by manipulation of the control systems.
- Plasmids can differ in their stability in a cell. A plasmid from a cell can be lost spontaneously or by manipulation.
- Two types of plasmids in a cell may be incompatible, resulting in the loss of one.
- Plasmids can be transferred from one cell (donor) to another cell (recipient), spontaneously or through manipulation.
- Plasmid transfer can occur either only between closely related strains (narrow host range), or between widely related strains from different species or genera (broad host range).
- A plasmid can be cryptic (i.e., not known to carry genetic code for a known trait).
- Effective techniques for the isolation, purification, and molecular weight determination of bacterial plasmids have been developed.
- Genetic codes from different sources (different procaryotes and eucaryotes) can be introduced into a plasmid, which then can be transferred in the cell of an unrelated bacterial species in which the phenotype may be expressed.

9.2.2 Some Characteristics of Small (about 10 kb) and Large (over 10 to about 150 kb) Plasmids

- **Copy number**—Small plasmids generally occur in multiple copies (10 to 40 per cell); large plasmids generally present in low copies (for a very large plasmid even one copy per cell).
- **Amplification**—Many small plasmids can be amplified to a very high copy number; large plasmids, especially very large ones, cannot be amplified.

- **Conjugal transfer**—Small plasmids are non conjugativc (but can be transferred along with a conjugative plasmid); large plasmids are generally conjugative.
- **Stability**—Small plasmids are usually unstable; large plasmids are usually stable; a small plasmid can only encode one or a few phenotypes.
- **Genetic code**—A large plasmid can encode many phenotypes. ⸳

9.2.3 Presence of Plasmids in Some Starter Culture Bacteria

Lactococcus species—Many strains from both subspecies and the biovar have been analyzed. Most strains have 2 to 10 or more types of plasmid, both small and large.

Str. thermophilus—Among the strains examined, a few strains carry plasmids, generally only one to three types; mostly small and not very large.

Leuconostoc species—Many species and strains carry plasmids of different sizes. ranging from 1 to 10 or note more ypes.

Pediococcus species—Limited studies have shown the strains carry from none to two to three plasmids; both small and large types.

Lactobacillus species—Only a limited number of species and strains have been tested. Some species carry plasmids very rarely, such as *Lab. acidophilus.* Some carry usually a few plasmids, such as *Lab. casei.*

Some carry a large number (two to seven) of plasmids of various sizes, such as *Lab. plantarum.*

Bifidobacterium species—Limited studies have revealed the species to harbour two to five different types of plasmids.

Propionibacterium species—Limited studies have shown some species to contain only a few plasmids.

9.2.4 Phenotype Assignment to a Plasmid

Following the understanding of plasmid characteristics and the revelation that many starter culture bacteria carry plasmids, studies were conducted to determine that a particular plasmid in a strain carries genetic codes necessary for the expression of a specific phenotype or to determine that a particular phenotype is linked to a specific plasmid in a strain. The loss of lactose fermenting ability (Lac$^+$ phenotype) among *Lactococcus lactis* strains was suspected to be due to loss of a plasmid that encodes gene(s) necessary for lactose hydrolysis (also for lactose transport). The possible linkage of Lac$^+$ phenotype to a plasmid in a *Lac. lactis* strain was first studied according to the following protocol:

1. A Lac$^+$ strain can become Lac$^-$ (inability to hydrolyze lactose) spontaneously, or when grown in the presence of a chemical curing agent (such as acriflavin) or at high temperature (physical curing agent).
2. Analysis of the plasmid profile (types of plasmid present as determined from their molecular weight in kilobase, kb) showed that a 45-kb plasmid, present in the Lac$^+$ wild strain, was missing in the Lac$^-$ cured variant. Thus, loss of this plasmid was correlated with the loss of Lac$^+$ phenotype in this strain.
3. To determine further that this 45-kb plasmid was really encoding the Lac$^+$ phenotype, the wild Lac$^+$ strain was conjugally mated with a plasmidless Lac$^-$ strain, and several Lac$^+$ transconjugants were obtained (this aspect is discussed

later for mechanisms of DNA transfer). When these transconjugants were analyzed, all were found to contain the 45-kb plasmid. Following curing, the transconjugants were converted to Lac⁻, and an analysis showed these variants no longer have the 45-kb plasmid

4. From these series of experiments, it was determined that the 45-kb plasmid in the specific *Lac. lactis* strain used encodes for the Lac⁺ phenotype.

Similar studies were conducted to determine plasmid linkage of several other traits in starter culture bacteria.

9.2.5 Plasmid-Linked Traits in Starter Culture Bacteria

Many starter culture bacteria, especially *Lac. lactis* strains, have been examined for the plasmid linkage of different phenotypes. These studies have revealed that in these bacteria, many commercially important traits are plasmid-encoded. Some are listed below.

- *Lac. lactis*

  Lac⁺, lactose hydrolysis (also lactose transport trait);

  Pro⁺, proteinase activity

  Cit⁺, citrate hydrolysis (also citrate transport trait)

  Bac⁺, production of several bacteriocins (also their respective immunity, processing, and translocation traits; bacteriocin, like nisin is encoded in a transposon)

  Phage^r, resistance to specific bacteriophages

  R/M system, restriction/modification

  Resistance to several antibiotics (such as Km^r, resistance to kanamycin)

  Metabolism of several carbohydrates (such as Gal⁺, galactose utilization)

  Muc⁺, mucin production

- *Str. thermophilus*—Plasmid linkage of a phenotype is not conclusively known

- *Leuconostoc* species—Bac⁺, production of different bacteriocins (also immunity against them)

- *Pediococcus* species:

  Suc⁺, sucrose hydrolysis

  Bac⁺, production of different bacteriocins (also immunity against them, processing, and translocation)

- *Lactobacillus* species—

  Lac⁺, lactose hydrolysis

  Mal⁺, maltose hydrolysis

  Bac⁺, production of some bacteriocins (also immunity against them)

  Muc⁺, ability to produce mucin

  Resistance to some antibiotics (such as erythromycin. Em^r)

  R/M system, restriction/modification

The same phenotype in a species can be encoded in different size plasmids.

9.3 GENE TRANSFER METHODS IN STARTER CULTURE BACTERIA

Once the genetic basis of a phenotype in bacteria was understood, studies were conducted to develop means to transfer the genetic materials from one bacterial cell to another. It is recognized that exchange of genetic materials occurs among bacteria naturally, but at a slower space. However, if a process of introducing genetic materials can be developed under laboratory conditions, the process of genetic exchange not only can be expedited, it can also help in developing desirable strains. In starter culture bacteria, this would help to develop a strain for a specific fermentation process that carries many desirable phenotypes and the least number of undesirable phenotypes.

Results of the studies conducted since the 1970s, initially in *Lac. lactis* subspecies and later in other lactic acid bacteria, revealed that genetic materials can be introduced into bacterial cells by several different mechanisms. Some of these are discussed here.

9.3.1 Transduction

In this process, a transducing bacteriophage mediates the DNA exchange from one bacterial cell (donor) to another cell (recipient). DNA of some phages (designated as temperate bacteriophages) following infection of a cell can integrate with bacterial DNA and remain dormant. When induced, the phage DNA separates out from the bacterial DNA and, on some occasions, can also carry a portion of the bacterial DNA encoding a gene or genes in it. When the phage-carrying portion of a bacterial DNA infects a bacterial cell and integrates its DNA with bacterial DNA, the phenotype of that gene is expressed by the recipient cell. Initially, the Lac$^+$ phenotype from a lactose-hydrolyzing *Lac. locus* strain carrying a temperate phage was transduced to a Lac$^-$ *Lac. lactis* strain to obtain a Lac$^+$ transductant. This method has been successfully used to transduce Lac$^+$ phenotype and several other phenotypes (such as Pro$^+$) in different strains of *Lac. lactis* subspecies. Investigation showed that both chromosomal and plasmid-encoded genes from bacteria can be transduced. Transduction has been conducted successfully in some strains of *Str. thermophilus* and in strains of several *Lactobacillus* species.

The transduction process in starter culture bacteria is important in order to determine location of a gene in bacteria for genetic mapping and to study its characteristics. Even though a temperate phage can be induced spontaneously, resulting in lysis of bacterial cells, it is not very useful in commercial fermentation. Also, this method cannot be applied in species that are known not to have bacteriophages, such as some *Pediococcus* species.

9.3.2 Conjugation

In this process, a donor bacterial cell transfers a replica of a portion of its DNA to a recipient cell. The two cells have to be in physical contact to effect this transfer. If the transferred DNA encodes for a phenotype, the transconjugant will have that phenotype. To make the DNA transfer possible, the donor cells should have several other genes, such as a clumping factor (for a physical contact through clumping) and a mobilizing factor (to enable the DNA to move from a donor to a recipient). The process consists of selecting the right donor and recipient strains, mixing the two cell types in a 2:1 to 10:1 (donor : recipient) ratio in several different ways for DNA transfer to occur, and then identifying the transconjugants by appropriate selection techniques.

This technique has been used successfully to transfer several plasmid-linked phenotypes in some lactic acid bacteria. The plasmid-linked Lac$^+$ phenotype was transferred conjugally between two *Lac. lactis* species. The transconjugant was Lac$^+$ and had the specific plasmid, loss of which resulted in its phenotype becoming Lac$^-$. Subsequently, the Lac$^+$ phenotype located in different plasmids in many *Lac. lactis* subspecies and strains were conjugally transferred to Lac$^-$ strains of the same species. Conjugal transfer of different plasmid-linked traits have also been reported in other lactic acid bacteria, such as the diacetyl production trait in *Lac. lactis* subsp, *lactis* biovar diacetilactis.

The method has several limitations, some of which have been listed previously with the characteristics of plasmids. These include plasmid size, plasmid incompatibility and instability in recipient strains, the inability to express in hosts, the inability to have the proper donors and recipients, and in some cases, the inability to recognize the transconjugant. However, using a broad host range plasmid (e.g., pAMβ1, a plasmid of *Enterococcus* species encoding antibiotic gene), it was possible to show that plasmid transfer by conjugation is possible among lactic acid bacteria between some species, between two different species in the same genus, or between two different species from different genera.

9.3.3 Transformation

The method involves extraction and purification of DNA from a donor bacterial strain and mixing the purified DNA with the recipient cells. The expectation is that some DNA can pass through the cell barriers (wall and membrane) and become part of the host DNA expressing the new phenotype. In some Gram-positive bacteria (e.g., *Bacillus* species), this method has been effective for the transfer of certain traits. In lactic acid bacteria, limited studies revealed the technique to be not very effective. However, a modified method was found to be effective. First *Lac. lactis* cells were treated with lysozyme and/or mutanolysin to remove the cell wall and to form protoplasts in a high osmotic medium. The protoplasts were then exposed to purified DNA (chromosomal, Plasmid, or phage DNA) in the presence of polyethylene glycol. The growth conditions were then changed for the protoplasts to regenerate cell wall. The transformants were then detected in a selective medium. By this method, *Lac$^+$* phenotype and Em^r (erythromycin resistance phenotype) and phage DNA (transfection) were transferred to recipient strains of *Lac. lactis* subspecies. Due to limitations in the success rate, this method is not widely studied in lactic acid bacteria.

9.3.4 Protoplast Fusion

The technique involves preparation of protoplasts of cells from two different strains and allows them to fuse together in a suitable high osmotic environment. Fusion of cells of the two strains and recombination of the genetic materials may occur. Allowing the protoplasts to regenerate cell wall and using proper selection techniques, recombinants carrying genetic information from both strains can be obtained. It has been used successfully to produce recombinants of *Lac. lactis* subspecies for both Lac$^+$ and Em^r phenotypes. However, this technique is not used very much in lactic acid bacteria.

9.3.5 Electrotransformation

In this method a suspension of recipient cells in high population levels (10^8 cells per 200 µl) is mixed with purified DNA from a donor strain and then exposed to a high-voltage electric field for a few microseconds. This results in temporary formation of small

holes in the cell barrier (membrane) through which purified DNA can pass. Subsequently, the cells were allowed to repair their damage and express the new phenotype to enable their isolation.

This method has been widely used in many lactic acid bacteria to introduce plasmids from the different strains of the same species and from separate species and genera. In addition, vectors carrying cloned gene(s) from other sources have been successfully introduced in several species of lactic acid bacteria. This is currently the most preferred method used to transfer DNA from a source into the recipient cells of lactic acid bacteria.

9.3.6 Gene Cloning

In the simplest form, in this technique, a DNA segment, carrying a gene or genes, is first obtained by digesting the purified DNA of the donor with the suitable DNA restriction endonuclease(s) and purifying it from the mixture. A suitable plasmid (cloning vector) is selected that has one or several gene markers (such as resistance to an antibiotic) and a site that can be digested with the same restriction enzyme(s). The plasmid is digested with this enzyme, mixed with the donor DNA fragment and incubated for the fragment to align in the opening of the plasmid DNA. The open ends are then sealed using suitable enzyme(s). This plasmid carrying the gene(s) from the donor can then be introduced in a bacterial cell in several different ways, most effectively by electroporation in lactic acid bacteria, as described above. This method is now being studied to transfer genes from different sources into lactic acid bacteria.

9.4 GENETIC MODIFICATION OF LACTIC ACID BACTERIA USING RECOMBINANT DNA TECHNIQUES

Approaches using recombinant DNA techniques have led to most of the recent advances in understanding the physiological characteristics of lactic acid bacteria; these are being used to construct strains with unique industrially significant characteristics. The advantage of using recombinant DNA approaches for studying physiology is that strains can be constructed that differ in a single defined genetic alteration, such as inactivation of a specific gene. By comparing the wild-type culture to its isogenic derivative, the role of that gene in the phenotype being examined can be unequivocally determined. This general approach has resulted in a detailed understanding of how these bacteria use lactose, obtain essential amino acids, produce diacetyl, and resist bacteriophage infection.

Use of recombinant DNA approaches holds great promise for the construction of dairy starter cultures with enhanced industrial utility. The inactivation or over expression of genes in a metabolic pathway can be used to alter end products that accumulate from a given pathway. One example of such an approach is described for citrate metabolism. Regulated promoters can be used to express genes in a controlled manner (Kok, 1996). One application of such promoters is the controlled expression of lytic genes resulting in autolysis of the starter culture. This would result in rapid release of enzymes (i.e., peptidases) into the cheese matrix and potentially accelerate cheese flavour development. Several recombinant DNA approaches have been applied to constructing starter cultures with novel bacteriophage-resistance mechanisms (Dinsmore and Klaenhammer, 1995). These include high copy presentation of native phage defense systems (restriction/modification systems

and abortive infection systems), antisense RNA, cloning of bacteriophage origins of replication on plasmids, and construction of a phage-inducible suicide system.

Of these approaches, the phage-inducible suicide system appears to have the greatest potential for the construction of starter cultures with enhanced resistance to infection by bacteriophage. The system consists of a phage-inducible promoter that activates a lethal gene after bacteriophage infection (Djordjevic *et al.*, 1996). The system presents a genetic trap, wherein the objective is to invite phage infection and then trigger a lethal device that destroys the host bacterium and phage, before progeny phages are replicated within the culture. In the single construct described to date, the phage-inducible promoter was isolated from a bacteriophage, and a restriction endonuclease cassette was used as the lethal gene of the suicide system.

Recently, there has been considerable interest in the application of genetic engineering to improve existing strains or produce new strains of lactic acid bacteria for use in starter cultures. Attributes considered for improvement are:

(i) rapid acid production,

(ii) bacteriophage resistance,

(iii) proteinase activity

(iv) absence of bitter flavours,

(v) general flavour enhancement,

(vi) ability to produce bacteriocins, etc.

Despite rather slow progress, promising applications of recombinant DNA technology to dairy product manufacturing are envisioned through the improvement of the starter cultures. Although genetic manipulation of these dairy cultures is now being carried out extensively by several group of workers all over the world, major commercial application of these modified organisms are still to be explored fully as a lot of work still needs to be carried out at the fundamental level to understand their genetic make-up and control.

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10

INTRODUCTION TO FERMENTED MILKS

10.1 INTRODUCTION

The major **advantages of fermented milks** include their relatively easy production, better keeping quality, better nutritive value, easier digestibility, processing pleasing acid and flavour attributes and strong therapeutic potentials. Due to these advantages, currently, about 2.3% of the total dairy product market is in the form of cultured dairy products and their production has been showing a marked increase. With the advent of biotechnological techniques used for the genetic manipulation of lactic acid bacteria to improve their fermentative characteristics, the popularity and consumption of these high-value products have increased dramatically during the last couple of decades all over the world. A variety of such products are now available for consumption for a wide section of consumers depending upon the individuals's liking. As a result of better understanding of their beneficial effects on human nutrition and health, fermented milk products are now in great demand throughout the world. Various aspects of fermented milks are briefly discussed in this chapter.

10.2 DEFINITION AND COMPOSITION OF FERMENTED MILKS

As per IDF specifications, fermented milks are defined as "the products prepared from milks—whole, partially or fully skimmed, concentrated milk or milk substituted from partially or fully skimmed dried milk, homogenized or not, pasteurized or sterilized and fermented by means of specific organisms."

Fermented milks contain the constituents as present in initial milk, except that lactic acid and other metabolities will increase. The typical composition of fermented milk as per IDF (1981) is shown in Table 10.1.

Table 10.1 : Typical composition of fermented milk as per IDF (1981)

Constituent	Level (%)
Dry matter	14-18
Protein	4-6
Fat	0.1-10.0
Lactose	2-3
Lactic acid	0.6-1.1
Carbohydrates	5-25
pH	3.8-4.6

The composition of fermented milks vary over wide range depending on several factors, e.g. depending upon the initial composition of milk and the specific metabolic activity of starter cultures.

10.3 CLASSIFICATION AND TYPES OF FERMENTED MILKS

The fermented milks are classified, according to the nature of fermentation and acid content, into following four types:

Type	Examples
(1) Acid-alcohol	Kefir, kumiss
(2) High-acid	Bulgarian sour milk
(3) Medium-acid	Acidophilus milk, yoghurt
(4) Low-acid	Cultured buttermilk, cultured cream

Fermented milks appear under various names in different parts of the world. The names identify the country or region where they are originated/produced. Most of the naturally fermented milks contain more than one type of bacterium and occasionally yeasts because the characteristic flavours are produced through the growth of microbial consortia (a mixture of different strains of bacteria and may be yeasts) present naturally in the environment. The existence of diverse types of fermented milks, which are produced by and large with similar starter cultures and yet differing very much from each other is ascribed to the large spectrum of metabolic activity and specificity of strains used under different conditions with the preponderance of lactic acid bacteria as well as yeasts and molds in most of the traditional fermented milks.

Some important features of various types of fermented milks are summarized in Table 10.2.

Table 10.2 : Some examples of fermented milk products

Fermented product	Major responsible Microorganisms	Country of origin and popularity	Source of milk
Acidophilus milk	*Lb. acidophilus*	USA, Australia	Bovine
Buttermilk	*Lc. cremoris*	USA, Scandinevian	Bovine, Buffalo
	Lc. diacetylactis	and European countries	

... contd.

Fermented product	Major responsible Microorganisms	Country of origin and popularity	Source of milk
Cottage cheese	*Lc. cremoris* *Lc. lactis*	USA	Bovine
Cultured cream	*Lc. lactis* *Lc. cremoris* *Lc. diacetylactis* *Leuc. Cremoris*	USA	Bovine
Cream cheese	*Lc. cremoris* *Lc. lactis* *Lc. diacetylactis* *Leuc. Cremoris*	USA	Bovine
Koumiss	*Lb. bulgaricus* *Torulopsis holmii* (yeast) *Sacch. lactis*	Russia, Asia	Horse, Mare, Camel
Kefir	*Lb. brevis, Lb. lactis* *Lb. casei, Lb. delbrueckii* *Lb. caucasicus* *Leuc. mesenteroides* *Lc. durans, Torulaspora delbrueckii* (yeast) *Sacc. cerevisiae* (yeast) *Acetobacter aceti*	Russia	Bovine, ewe, goat, or mixed milk
Labneh	*Lb. thermophilus* *Leuc. lactis* *Lb. acidophilus* *Kluyveromyces fragilis* (yeast) *Sacc. cerevisiae* (yeast)	Israel, Arab countries	Bovine, Sheep Goat
Laktofil	*Leuc. cremoris* *Lc. cremoris* *Lc. lactis* *Lc. diacetylactis*	Sweden	Bovine
Leben	*Lb. bulgaricus* *Lc. thermophilus* *Lc. lactis*	Iraq, Labenon	Ewe, goat, sheep

... contd.

Fermented product	Major responsible Microorganisms	Country of origin and popularity	Source of milk
Leben raib	As for Leben	Egypt	Bovine, buffalo
Long milk	*Streptococcus* sp. *Leuconostoc* sp.	Scandinavia	Bovine
Quark	*Leuc. cremoris* *Lc. cremoris* *Lc. lactis* *Lc. diacetylactis*	Germany/ Europe	Bovine
Viili	*Streptococcus* sp. *Leuconostoc* sp.	Finland	Bovine
Yakult	*Lb. casei*	Japan	Bovine
Yogurt	*Lb. delbrueckii* ssp. *bulgaricus* *Lc. thermophilus*	Turkey. Caucasus Middle Asia	Bovine, Goat or Mixed milk
Indian/Indigenous			
Dahi	*Lb. lactis* *Lb. bulgaricus* Lactose fermenting Yeasts (Mixed culture)	India, Persia	Bovine, buffalo
Srikhand (Chakka)	*S. salivarius* *Lb. bulgaricus*	India	Bovine, buffalo
Lassi	*S. salivarius* *Lb. bulgaricus*	India	Bovine, buffalo

10.4 PRODUCTION OF FERMENTED MILKS

10.4.1 Traditional Method

The traditional method for the production of fermented milk was by natural fermentation of surplus milk collected on the day. During the harvesting of the milk, microorganisms on the utensils, from the animals and from the environment find their way into the milk. The environmental conditions select bacteria that ferment the milk sugar, lactose. When the harvested milk leaves the animal its temperature is about 37°C and if it is not cooled quickly to about 4°C the contaminating bacteria grow in the milk. Traditionally, due primarily to the lack of proper temperature control, this resulted in sour milk that was tastier, possessed a thicker texture and had a longer shelf life than the fresh milk. Eventually in traditional societies the primitive but simple technique was developed of keeping back a small portion of the milk, whey, etc to add to the next day's supply of milk. It was a kind of culturing that is still used to this day for some fermentation applications.

10.4.2 Commercial Method

With the development of science of Microbiology the bacteria responsible for the unique characteristics of each type of fermented milk product were isolated and pure culture techniques applied to improve the quality of milk products.

The milk intended for fermented milk manufacture should be fresh, clean and free from developed acidity and any off flavours. It should be free from antibiotic residues and other inhibitory substances not destroyable by heat treatment. If such substances remain, they may adversely affect the performance of starter cultures. The initial microbial load in raw milk should also be less and it should not have been stored for longer time before processing. Treatments like filteration, clarification, Bactofugation, etc., can be applied to make the milk clean and microbiologically better.

Standardization is a usual process applied to raw milk for adjusting its fat and solids content. This is required, not only from commercial view point, but also to keep uniform quality product throughout and have desired body, texture, flavour characteristics in the product.

Standardization may require addition of skim milk powder, whey powder, water, cream, etc. The quality of such ingredients also should be monitored when used.

In certain cases, homogenization of milk is also done, which gives homogenous dispersion of fat and other constituents added in milk. It also improves viscosity, richness and digestibility of the product. However, homogenization breaks the clumps of bacteria, leading to apparent increase in plate count.

Then, the milk is required to be heat treated before inoculation with starter culture and followed by incubation at required specific temperature. Heat treatment upto 80-85°C for 30 min or 90-95°C for 10 min or boiling for 5 min are commonly used.

The typical flow diagram for the preparation of any type of fermented milk is generally followed as shown below—

Selection of Raw Milk
↓
Standardization of Fat and Solids Conents
↓
Suitable Heat Treatment
↓
Cooling to Optimum Temperature
↓
Inoculation with required Starter Cultures
↓
Incubation at specific temperature for required period
↓
Fermentation
↓
Setting
↓
Cooling
↓
Fermented Milk
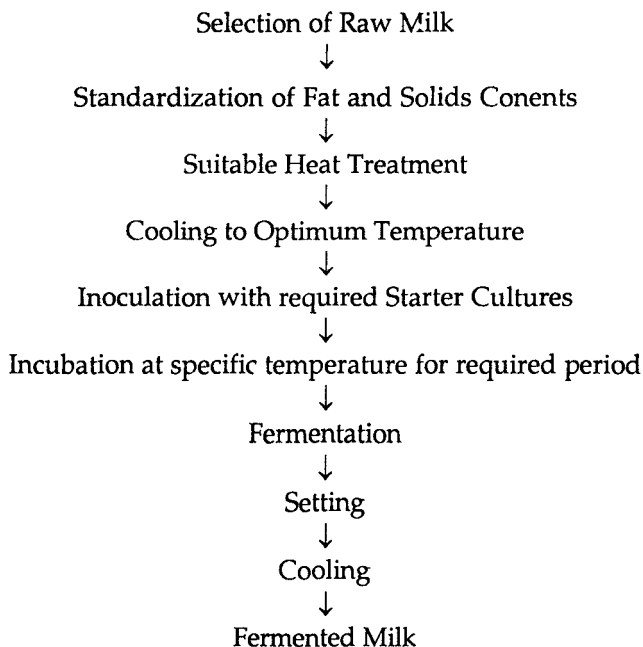
10.5 NUTRITIONAL VALUE AND PROPERTIES OF FERMENTED MILK

The nutritional value of a food is dependent not only upon its nutritional content but also upon the availability, digestibility and assimilability of the nutrients. Cultured dairy products have the same caloric value as the milk from which they are made, yet are more nutritious because of their ease of digestion, higher concentration of enzymes and increased level of B-vitamins.

Yoghurt proteins have a higher *in vitro* digestibility (Breslaw & Kleyn 1973) and higher biological value (Rasic *et al.*, 1971; Simhaee & Keshavarz 1974) than milk proteins. The free amino acid content of yoghurt is also higher than in milk, due to the heat treatment and proteolysis by the yoghurt bacteria (Rasic *et al.* 1971). Several other studies on the proteolytic and lipolytic activity of various lactic cultures (Poznanski *et al.* 1965; Chandan *et al.*, 1969a,b) demonstrated the relative importance of the lactobacilli in the pre-hydrolysis of milk protein and the importance of streptococci and leuconostocs in the pre-hydrolysis of milk fat.

Hargrove & Alford (1978) also reported that rats had better weight gains and higher food efficiency when fed yoghurt as compared with milk or other fermented or acidified milks. The yoghurt diet maintained superiority even when the other diets were supplemented with vitamins.

Lactose, the carbohydrate in milk, causes nutritionally related problems in some persons who are deficient in the intestinal enzymes lactase or β-galactosidase. These people, therefore, must restrict their dietary intake of milk and milk products. Infants, children and adolescents require the calcium in milk for proper bone growth and development, and a severe restriction in the intake of dairy products thus has serious nutritional consequences. Therefore, it has been suggested that lactose-intolerant persons consume yoghurt and other cultured dairy products because up to half the lactose of the milk is hydrolysed by the lactic organisms during the manufacturing process (Gallagher *et al.* 1974). Such products may also supply additional quantities of the enzyme lactase, which is elaborated by the microbial starter cultures.

Goodenough & Kleyn (1976) noted that dietary yoghurt containing viable organisms greatly enhanced the lactase activity of rat intestinal mucosa. The effect was attributed to inherent lactase activity in the yoghurt, since no comparable effect was noted in rats fed either pasteurized yoghurt or simulated yoghurt containing sucrose or lactose.

Kilara & Shahani (1976) also demonstrated that the level of lactase increases during the incubation of yoghurt cultures. Subsequent *in vitro* digestion of the yoghurt released the enzyme from the culture cells. Since the lactase can be released from the yoghurt by digestion and as it appears to enhance intestinal lactase activity, it is possible that yoghurt could be of benefit to persons with lactose intolerance.

While many lactic organisms require B-vitamins for growth, several cultures are capable of synthesizing vitamins. The extent of biosynthesis or utilization of B-vitamins by the lactic organisms depends on the temperature, length of incubation and other processing parameters (Nilson *et al.* 1965).

The bioavailability of essential minerals like Ca, P, Cu, Mg, etc. is also increased in fermented milks.

Due to all these nutritional properties, fermented milks give better growth rate and show high feed efficiency.

10.6 THERAPEUTIC PROPERTIES AND VALUES OF FERMENTED MILKS

In addition to the superior nutritional quality of fermented milks, there are other important attributes affecting the health of the consumer.

(1) When starter cultures are growing in milk, they synthesize a number of **antimicrobial compounds** like lactic acid, acetic acid, hydrogen peroxide, diacetyl etc. These compounds with conditions of low pH and Eh, exert bacteriostatic or bactericidal effect on many human pathogens.

The competitive antagonism in the intestinal tract by specific starters like *Lb. acidophilus* suppresses pathogens. The antibacterial activity differs with different starter organisms, but in general it has been found to inhibit *E. coli*, *Salmonella* spp., *Shigella* spp., *B. cereus*, *Staph. aureus*, *Ps. aeruginosa*, *List. monocytogenes*, *Candida albicans* and some other pathogens.

Fermented milks have been used in cases of diarrhoea, colitis, constipation, flatulence, dyspepsia and gastroenteritis.

(2) Several lactic organisms produce natural antibiotics called *bacteriocins*. For example, *Strep. lactis* produce *nisin*, *L. bulgaricus* produces *bulgarican* and *L. acidophilus* produces a number of antibacterial compounds which have been called *acidolin, acidophilin* and *lactocidin*. All these possess inhibitory activities against a wide variety of Gram positive and Gram negative pathogens.

(3) The organisms able to implant in the intestinal tract, when present in fermented milks help in regulation of balance of intestinal flora.

(4) It is also useful in replenishing the intestinal tract by beneficial microflora, especially in post-operative cases, when high dozes of antibiotics might have destroyed the intestinal flora.

(5) As fermented milks are rich in enzyme beta-galactosidase, it helps to digest lactose in lactose-intolerant people. This aspect is elaborately discussed in above section of nutritional values and properties of fermented milks.

(6) Certain lactic cultures have been found to be useful in control of coronary heart diseases due to their hypocholesterolemic effect. They can reduce serum cholesterol levels either by assimilation or indirectly lower the pool through deconjugation of bile acids.

Hepner *et al.* (1979) reported a hypocholesterolemic effect in human subjects receiving a one-week dietary supplement of yoghurt.

(7) In last few years the work on anticarcinogenic properties of fermented milks have increased. Several trials have indicated that they can control tumour growth and reduce the chances of colon cancer.

(a)   Bogdanov *et al.* (1962) were perhaps the first to observe that *L. bulgaricus* possessed a potent antitumour activity. Later, they isolated three glycopeptides which have promising biological activity against Sarcoma 180 (Bogdanov *et al.* 1975).

In a study in collaboration with Sloan-Kettering Institute for Cancer Research, several lactobacilli were tested against Sarcoma 180, and *L. acidophilus* DDS 1 possessed definite antitumour activity.

(b)   Shahani and Friend, using Ehrlich ascites tumour cells, showed that extracts of *L. acidophilus* and yoghurt possessed antitumour activity. When colostrum fermented with *L. acidophilus* DDS 1 was fed to mice, there was inhibition of tumour proliferation ranging from 13 to 38% (Bailey and Shahani 1976).

(c)   Reddy *et al.* (1973) and Farmer *et al.* (1975) found that mice given yoghurt displayed from 28 to 35% inhibition of ascites tumour. Feeding milk, lactose, lactic acid or cells killed by heat showed no inhibitory effect.

(8) Certain fermented milks increase specific or non-specific immune response. Certain products like kefir and kumiss are used in treatment of tuberculosis since long.

(9) Fermented milks do not have any adverse effects. So they are recommended, not only for patients but for healthy individuals also, because they help in maintaining health and vigour.

10.7 ASSESSING QUALITY OF FERMENTED MILKS

Like any other food product, the preparation of fermented milks also requires strict adherence of good manufacturing practices in order to obtain an organoleptically and hygienically sound product. For fermented milk products, strict compliance to the hygienic recommendations of good manufacturing practices alongwith good technological practices must be followed in order to achieve full control of the entire production process and detection of the risk areas where faults may occur. In case of fermented milks, faults are mainly due to incorrect fermentation, gel/curd treatment and recontamination during handling.

Hence, if good manufacturing practices are followed correctly and completely throughout the process, limited monitoring will be sufficient to assess the product safety and quality and thus consumer acceptability. In this regard, the following precautions need to be strictly followed to guarantee the production of high quality, nutritious, wholesome and safe fermented milks:

(i)   Correct cleaning and sanitization of room, plant and equipment.

(ii)   Use of superior quality (microbiological and chemical) raw materials.

(iii)   Adequate heat treatment of milk and ingredients.

(iv)   Use of pure selected starters of proven quality under aseptic conditions.

(v)   Fermentation process with controlled temperature and time.

(vi)   Gel breaking (stirred products) under constant conditions.

(vii)   Aseptic filling.

(viii) Hygienic packaging and storage.

10.7.1 Quality Control of Raw Materials and Starters

In order to produce high microbiological quality fermented milks, the raw materials and other ingredients must also be of high quality. The microbiological quality of these ingredients can be monitored by subjecting the samples to standard plate counts on the

appropriate media for assessing the microbial load. The microbiological quality of starters is also equally important for producing high quality standard fermented milks.

10.7.2 Microbiological Analyses of Fermented Milks

Microbiological tests are conducted to determine the number of specific starter bacteria used for making the fermented milk and also the contaminating microorganisms. Samples should be prepared as recommended. Counts of the microorganisms characteristic of each fermented milk like lactococci, lactobacilli, leuconostocs, etc. are made on different media. In case of fermented milks made with mixed cultures, the identity of the typical colonies on different media should be confirmed by microscopic observation and rapid identification procedures. All contaminating microorganisms including bacteria, lactose fermenting yeasts, etc. must also be looked for in the product. For the assessment of processing hygiene, it is worthwhile detecting the presence of coliforms, staphylococci and enterococci since these organisms may survive in low acid fermented milks (pH 5.0). In addition to this, the fermented products should also be examined for the presence of yeasts and molds as these highly undesirable organisms can thrive luxuriously in the acidic environment, prevailing in the fermented products.

BIS has suggested following standards for Dahi or Yoghurt in India—

(1) Titratable acidity = 0.7%

(2) Coliform count = <10/g

(3) Yeast and Mold count = <10/gram

10.7.3 Assessment of Sensory Characteristics

The sensory evaluation of fermented milks is virtually the same as done for all other food products. The progress in methods of sensory evaluation now allows for the classification, differentiation and comparison of fermented milks according to their organoleptic properties. The following characteristics of the products should be evaluated:

(i) General appearance (phase separation, gas bubbles, foam, granulation, foreign matter, etc).

(ii) Odour and aroma

(iii) Colour

(iv) Body and texture

(v) Taste and flavour

10.7.4 Public Health Safety Aspects

Like all other food products, fermented milks must also possess defined nutritional and hygienic qualities in order to protect the consumer's health and obtain the expected specific characteristics. The concept of food safety covers two different aspects:

(i) The product must guarantee that it contains all the specific nourishing factors peculiar to the product to satisfy the consumer's requirements and expectations.

(ii) The product must not be a danger to the consumer's health through the presence of physical, chemical and biological contaminants.

The wholesomeness of the fermented milks is generally guaranteed by their specific microbiological composition. Besides the nutritional factors of the raw materials and the metabolites of the starters, the presence of specific viable organisms assumes a nutritional relevance that guarantees health safety. This is mainly due to the production of lactic acid, antibiotics or bacteriocins and competing microflora of the gut. All of these factors do not allow the growth and survival of undesirable microorganisms, particularly the pathogens. Nevertheless, these fermented milks may sometime act as passive vehicles for residues of toxic substances or the infectious bacteria due to incorrect processing. Both these problems can be tackled by the rigorous selection of raw materials and packaging materials and by strict application of good manufacturing practices. Although, there is no report of toxic infections caused by fermented milks consumption, it is, however, true that pathogens may survive in the fermented milks for a variable period depending on the acidity level of the product.

Hence, it can be concluded that the objective of the quality control can be fully achieved only if the fermented products both while leaving the factory and when purchased from the market offer the consumer full guarantee regarding its nutritional value, wholesomeness and safety.

10.8 DEFECTS AND SPOILAGE OF FERMENTED MILKS

Fermented milks have average keeping quality of 1 to 3 weeks at refrigerated temperature. The major spoilage is through souring or growth of yeasts and molds due to their acidic nature. The defects seen in fermented milk also vary depending on the type and set or stirred state of the product. Table : 10.3 lists causes and remedies for common defects in fermented milks.

Table 10.3 : Defects in fermented milks

	Defect	Causes	Remedies
1.	Whey separation	Over or under acidfication, mechanical damage, low solids content, high incubation temp., insufficient heat treatment of milk, etc.	Homogenization and proper heat treatment of milk, keep low incubation temp., make use of slime producing organisms.
2.	Sour	Too high incubation, improper cooling	Check incubation and cooling conditions.
3.	Flat/Sweet	Too low incubation, cultures slow, improper avoid contamination development of acid and aroma producers, contamination	Use active cultures in desired proportion.
4.	Gassiness	Over growth of gas producers, yeast or	Ensure purity of cultures and prevent

... contd.

	Defect	Causes	Remedies
		coliform contamination	contamination during product manufacture.
5.	Unclean, rancid cheesy, yeasty, bitter, fruity flavours	Contamination by proteolytic & lipolytic flora.	Check contamination.
6.	Ropiness	Ropy strain of cultures dominating, too low temperature of incubation, contaminants	Ensure proper control of ropy strain if used, check contaminants, use proper incubation temperature.
7.	Moldy	Mold growth	Control molds
8.	Coloured spots	Pigment producing contaminants growing on top, mold growth.	Effective control of contaminants

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FERMENTED MILK PRODUCTS

11.1 INTRODUCTION

In Indian subcontinent, the conservation of milk by souring with the leftover of previous day sour milk has been a common practice ever since the Aryans inhabited the land. In this way, the shelf-life and utility of milk nutrients were extended. Today, the practices of preserving milk by fermentation has become a fascile household technology and fermented milks have become almost a compulsory dietary adjunct in this part of the subcontinent. **Dahi**, the indigenous fermented milk invariably figures as a regular item in our menu. Later, several other types of fermented milk products like yoghurt, acidophilus milks, kefir, kumiss, cheese, srikhand and others made their appearance among the multitude of fermented milks, which are elaborately discussed in this chapter.

11.2 ACIDOPHILUS MILK

Therapeutic and health promoting attributes of acidophilus milk have been known since long. It is also claimed that *L. acidophilus* can be established in the intestine of an adult and that of a diseased person afflicted with various gastrointestinal disorders like constipation, diarrhoea, etc. and hence can help in combating these disorders. Following this discovery, considerable amounts of L. *acidophilus* were distributed mostly in the form of milk culture to control gastric disorders. At present, this product is consumed in metropolitan cities of different countries for relief of intestinal disorders on the advice of physicians.

(a) **Definition**—Acidophilus milk is defined as highly acidic product made by fermentation of milk with *Lactobacillus acidophilus*.

(b) **Method of manufacture**—The flow diagram showing the steps involved in the manufacture of this product is given in the ensuing illustration (Fig. 11.1).

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        ┌─────────────────────────┐
        │    Receiving Milk        │
        │ (Skim/Whole/Defatted)    │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │ Filtration/Clarification │
        │      (35°-40°C)          │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │     Homogenization       │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │      Sterilization       │
        │    (115°C/15 min)        │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │    Cooling (38°-40°)     │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │    Inoculation (3-5%)    │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │       Incubation         │
        │   (38°-40°C/12-16 hr)    │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │      Coagulation         │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │  Break-up of Coagulum    │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │     Cooling (10°C)       │
        └───────────┬─────────────┘
                    ↓
        ┌─────────────────────────┐
        │ Packaging and storage (5°C)│
        └─────────────────────────┘
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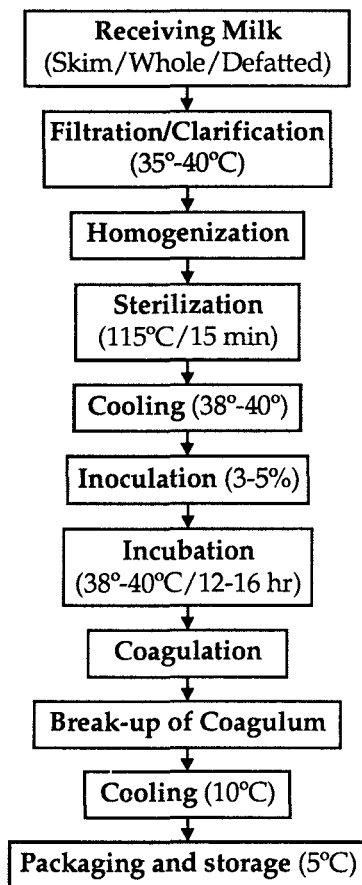
Fig. 11.1 : Method of manufacture of acidophilus milk.

(c) Composition—

Lactic acid	:	1.0% (0.6-0.7% for medicinal use)
Lactobacillus counts	:	2,000- 3,000 million/ml

(d) Therapeutic value of acidophilus milk—Acidophilus milk is known widely more for its possible therapeutic value than for its flavour. The use of this fermented milk as a therapeutic agent is based on the assumption that this formulation combats the so-called auto-intoxications caused by accumulation in the body of toxic substances elaborated by the growth of certain toxigenic bacteria. In addition to this, the constant use of acidophilus milk is recommended for controlling several other intestinal disorders. The proper use of acidophilus milk in several feeding trials substantiated the beneficial effect of this product in patients suffering from simple constipation, constipation accompanied by bilary symptoms, so-called "mucous-colitis" or irritable colon, and colonic ulcerative colitis.

In a study conducted in the United States, it was shown that feeding L. *acidophilus* milk to rats for 4 weeks significantly lowered the serum cholesterol level as compared to rats fed on either milk or water. However, no significant differences in the weight gain, feed intake, liver lipid levels and faecal lactobacilli counts were observed between the different

groups indicating that factors influencing serum cholesterol level were produced during fermentation of the acidophilus milk. *Lactobacillus acidophilus* is considered as a normal inhabitant of the intestinal tract and acidophilus milk has been considered as a practical means of bringing about a predominance of these organisms in the intestine. However, not all the strains of *L. acidophilus* can be established with equal success in the intestinal tract of man. Since, the therapeutic value of acidophilus milk is dependent upon the implantation of viable *L. acidophilus,* some physicians prescribe the regular use of acidophilus milk along with a suitable carbohydrate such as lactose or dextran. These carbohydrates are effective as they are absorbed from the intestinal tract fairly slowly, thus remaining available longer in the intestine as food for the lactobacilli. The ability of *L. acidophilus* to grow at low surface tension may account for its implantation in the gastrointestinal tract, whereas closely related species cannot grow there.

(e) **Microbiological quality of acidophilus milk**—*Lactobacillus acidophilus,* used as a starter in the preparation of acidophilus milk, is unable to multiply rapidly in milk and is easily overgrown by contaminating organisms. Moreover, the *L. acidophilus* cells lose their viability in the product, thereby, hampering their proper establishment in gastrointestinal tract for therapeutic gains. In other words, the two major microbiological problems faced during the manufacture of acidophilus milk are, how to avoid contamination of the culture and to keep the cells of L. *acidophilus* alive until they are consumed. Contamination can be avoided by strictly following the sterilization protocol for milk and by following standardized bacteriological procedures while transferring the culture. The organisms can be kept alive successfully, if acidity is not allowed to exceed 0.6-0.7% and storage temperature is maintained between 5-20°C. Under these conditions, *L. acidophilus* can be maintained for at least one week. For a longer storage, 0.65% acidity and 16°C storage temperature are recommended. The quality of acidophilus milk should be creamy with a mild acidic taste with no abnormal flavours and odours. It should be examined microscopically or culturally from time to time to ensure the purity of the product as well as the culture. In this way, the efficiency of the process can be checked.

(f) **Acidophilus based products**—Since acidophilus milk has very unattractive flavour and consistency, the acceptability of the product as such has been very limited. To improve consumer acceptability of the product, it is desirable to develop acidophilus based products with improved palatability and taste. If this could be possible, then a product with both nutritional and therapeutic properties comparable to that of the acidophilus milk could be made available to a broader section of the consumers especially in developing countries like India. A few such products which have already been developed are sweet acidophilus milk, acidophilus drink, acidophilus ice-cream and mixed culture yoghurt made with conventional yoghurt cultures and *Lactobacillus acidophilus* as well as acidophilus paste as listed in Table : 11.1.

Table 11.1 : Acidophilus products

	Name of the product	Organisms involved	Principle of processing
1.	Acidophilus sour milk	*Lb. acidophilus*	Fermentation
2.	A-38	*(Lc. lactis* + *Leuconostoc* spp.) + *Lb. acidophilus*	Both groups are fermented separately and mixed

... contd.

	Name of the product	Organisms involved	Principle of processing
3.	ACO-yoghurt	Yoghurt cultures +*Lb. acidophilus*	Both groups are fermented separately and mixed.
4.	Acidophilus yoghurt	Yoghurt cultures +*Lb. acidophilus*	Fermentation
5.	Acidophilus bifidus yoghurt	Yoghurt cultures + *Lb. acidophilus* + *B. bifidum*	Fermentation
6.	Bioghurt	S. *thermophilus* + *Lb. acidophilus*	Fermentation
7.	Bifighurt	S. *thermophilus* + *B. bifidum*	Fermentation
8.	Biogarde	S. *thermophilus* + *Lb. acidophilus* + *B. bifidum*	Fermentation
9.	Sweet acidophilus milk	*Lb. acidophilus*	Non fermented, added in chilled pasteurized milk.
10.	Acidophilus yeast milk	*Lb. acidophilus* + Lactose fermenting yeast	Acid & Alcohol fermentation
11.	Acidophilin	*Lb. acidophilus* + *Lc. lactis* + *yeast*	Mixed acid & alcohol fermentation.
12.	Acidophilus ice-cream	*Lb. acidophilus*	Ice-cream added with concentrated *Lb. acidophilus* before freezing.
13.	Acidophilus paste	*Lb. acidophilus*	Concentrated after fermentation by centrifugation.
14.	Dried acidophilus (powder, tablets, capsules)	*Lb. acidophilus*	Drying concentrated cells in milk based media by spray drying, vacuum drying or freeze drying
15.	Acidophilus whey	*Lb. acidophilus*	Fermented whey

11.3 BULGARIAN SOUR MILK

Bulgarian milk undoubtedly is a product belonging to yoghurt family. It was supposed to originate from Traki's tradition, i.e. from the tradition of sheep breeders, who came from Asia to Bulgaria in fifth century bringing the tradition of sour milk preparation, currently followed in Bulgaria. Bulgarian milk is an extremely sour milk prepared from boiled goat's or cow's milk inoculated with a portion pf previously fermented milk. The ripening is carried out at 40-45°C and the product is prepared in the same manner as yoghurt. At present, the commercial production of Bulgarian milk uses *L. delbrueckii* subsp. *bulgaricus* as a single culture or mixed with S. *thermophilus*. Incubation is done at 37°C and the level of milk acidity is kept at 1.4%. In some cases, acidity may reach as high as 4%. The soured milk is stored at 7°C. The traditional Bulgarian milk lacks flavour and aroma. Some people produce Bulgarian butter milk with the cultures, of *L. delbrueckii* subsp. *bulgaricus* and the starters for butter milk. The microbiological quality of this product resembles that of yoghurt.

11.4 BULGARIAN BUTTER MILK

(a) **Definition**—Milk soured with *L. delbrueckii* subsp. *bulgaricus* is named as Bulgarian butter milk. It is marketed by this name in Bulgaria. It is more viscious and has a higher acidity than cultured butter milk and lacks aroma characteristics of a good butter-milk culture.

(b) **Method of manufacture**—The steps in the manufacture of this product are essentially the same as those described for cultured butter milk. However, these are modified to suit the growth requirements of *L. delbrueckii* subsp. *bulgaricus*.

(c) **Microflora**—*L. delbrueckii* subsp. *bulgaricus* is the sole starter culture for bulgarian butter milk. The mother culture is carried in sterile milk and is incubated at 37°C for 10-14 hr resulting into an acidity of 1.0% or more. The final bulk culture is handled in the same way except that milk is adequately heat-treated. Most consumers object to high acidity in the product. However, elevated incubation temperature may lead to defects caused by the growth of contaminating bacteria escaping heat treatment. Hence, use of high quality milk is recommended to minimize this problem. Some manufacturers mix Bulgarian butter-milk with cultured butter-milk to improve the stability and to increase the acidity slightly.

(d) **Microbiological quality**—Adequate precautions pertaining to maintaining the microbiological quality of this product should be taken and these are by and large similar to those explained in respect of cultured butter milk.

11.5 BIFIDUS MILK

(a) **Definition**—Bifidobacteria are normal inhabitants of intestinal tract of new borns and infants. They possess special therapeutic properties and increase the resistance of the infant to several disorders. The milk cultured with Bifidobacteria is called bifidus milk.

(b) **Preparation and Microflora**—The Bifidobacteria are strict anaerobes and slow growers in milk, hence it is difficult to prepare bifidus milk. However, the product is made from severely heat treated milk taking greater aseptic precautions and using high rate of inoculum. The species regularly employed are *B. bifidum* or *B. longum*. However, use of aerotolerant strains of *B. adolescentis* have been found to give better product.

In recent years, several humanized milks or baby foods have also been developed by making use of *Bifidobacteria* or *Lb. acidophilus*.

11.6 CULTURED BUTTER MILK

Like other fermented products, cultured butter milk is also very popular in ' Scandinavian and European countries. A similar product known by the name "chhachh" is equally popular in the Indian subcontinent. Scandinavian sour milk is a typical European variant. The manufacturing procedures in respect of these cultured butter milks are more or less similar, whatever the national differences in respect of the end product may be.

(a) **Definition**—Cultured butter milk is the fluid remaining after ripened cream or sour cream in churned into butter. It is prepared by souring true butter milk or more commonly skim milk with a butter starter culture that produces a desirable flavour and aroma.

(b) **Composition**—

Acidity (% LA) : 0.8 - 0.85

Fat (%) : 0.5-3

(c) **Method of manufacture**—The method of manufacture of cultured butter milk is presented in the flow diagram (Fig. 11.2).

(d) **Microflora of cultured butter milk**—The starter cultures employed for the preparation of cultured butter milk are generally of mixed types consisting of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis*, *L. lactis* subsp. *cremoris* and *Leuco. citrovorum*. However, in case of Bulgarian milk, *L. delbrueckii* subsp. *bulgaricus* is the single starter. *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* are responsible for acid production whereas *L. lactis* subsp. *diacetylactis* and *Leuconostoc citrovorum* are the primary sources of flavour and aroma. Normally, starter cultures are inoculated at the rate of 1-2% for the manufacture of cultured butter milk and it is important that the cultures are both balanced and active. Routine checks on the balance between these organisms is essential. The main function of lactococci in the butter milk starter is to produce lactic acid which is necessary to give the desired sour taste, to curdle the milk and to lower the pH to the point where aroma bacteria can produce maximum amounts of volatile acids and, neutral products. *Leuconostoc citrovorum* functions chiefly by producing acetic acid, acetyl methyl carbinol and diacetyl from citric acid.

(e) **Microbiological qualify of cultured butter milk**—In order to produce the desired flavour and aroma in the butter milk, all the organisms in the starter must grow actively and must be present in sufficient numbers to bring about desirable changes. Most consumers prefer a butter milk that is not too viscous and has a smooth consistency with no whey separation. The best product is obtained after a fermentation time of 16-20 hr at 20°C. Insufficient flavour is associated with deficiencies in the starter cultures but the most frequent complaint concerns the physical separation. These defects can be tackled by vigorous and routine testing of the starter cultures (purity and activity) as well as by incorporating gelatin or additional fat into the processed milk. Utmost care should be taken to minimize microbial contamination during the processing of cultured dairy products.

(f) **Defects**—The most common defect of cultured butter milk is its poor stability which results in whey separation. This can be prevented either by adding gelatin or fat to milk or 10% milk culture of *L. delbrueckii* subsp. *bulgaricus* to the cultured butter milk. Another common defect is insufficient flavour and aroma developments due to loss of activity of the starters. The lower level of citric acid in milk (0.07%) may also account for insufficient flavour production. Hence, incorporation of 0.1-0.2% citric acid to milk before inoculation is recommended. Too little acid production may be attributed to inactive starter or presence of

inhibitory substances in the milk. Bacteriophages can also inhibit or prevent acid production in butter milk. Hence, use of phage insensitive strains of lactic starters is advocated.

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                    ┌─────────────────────┐
                    │ Receiving Skim Milk │
                    └─────────────────────┘
                              ↓
                         ┌───────────┐
                         │ Filtration │
                         └───────────┘
                              ↓
                    ┌─────────────────────┐
                    │   Pasteurization    │
                    │  (82°-88°C/30 min)  │
                    └─────────────────────┘
                              ↓
                      ┌─────────────────┐
                      │ Cooling (22°C)  │
                      └─────────────────┘
                              ↓
                      ┌──────────────────┐
                      │ Inoculation (1-2%) │
                      └──────────────────┘
                              ↓
                    ┌────────────────────┐
                    │ Incubation (21°-22°C) │
                    └────────────────────┘
                              ↓
                    ┌─────────────────────┐
                    │ Coagulation (12-16 hrs) │
                    │   (0.80-0.90% LA)   │
                    └─────────────────────┘

         Creaming

                    ┌──────────────────────┐
  Adding            │ Break-up of Coagulum │
  Butter            └──────────────────────┘
  Granules          ┌──────────────────────┐
                    │  Cooling (5°-10°C)   │
                    └──────────────────────┘
                              ↓
                       ┌──────────────┐
                       │ Butter Milk  │
                       └──────────────┘
                              ↓
                ┌───────────────────────────────┐
                │ Packaging and storage (5°-10°C) │
                └───────────────────────────────┘
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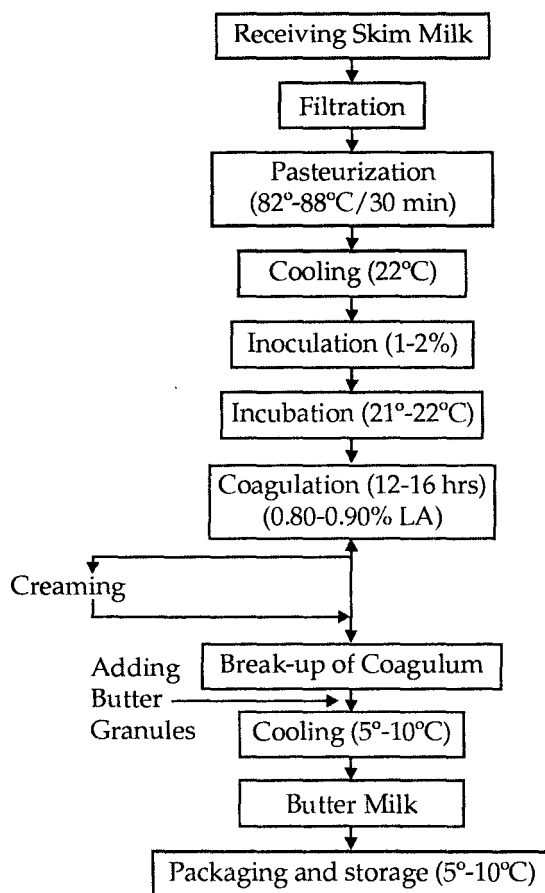
Fig. 11.2 : Flow diagram for manufacture of cultured butter milk.

11.7 CULTURED SOUR CREAM

(a) **Definition**—Cultured sour cream is an extremely viscous product with the flavour and aroma of butter milk but with a fat content of 12- 30%.

(b) **Method of manufacture**—This product is made in a manner similar to that of cultured butter milk. Ordinarily, it is consumed as a dressing or topping on other foods such as fruits. Typical schedule for production would involve various steps indicated in the Fig. 11.3.

(c) **Microbiological quality**—Since a heavy body is desired in the final product, agitation of the ripened cream must be kept to the minimum. In order to increase the viscosity, cooling and ageing at 5°C for several hrs is practised. The addition of dry milk solids to the original cream can also increase the consistency of the cultured products. The microbiological aspects pertaining to the quality of cultured cream resembles more or less with those of cultured butter milk.

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┌─────────────────────────────────────┐
│  Fortification of whole Milk with Cream │
│            (20% Fat)                  │
└─────────────────────────────────────┘
                  ↓
        ┌──────────────────────┐
        │       Heating        │
        │    (80°C/30 min)     │
        └──────────────────────┘
                  ↓
        ┌──────────────────────┐
        │    Homogenization    │
        │ (2,000 psi at 60°-80°C) │
        └──────────────────────┘
                  ↓
          ┌──────────────────┐
          │  Cooling (21°C)  │
          └──────────────────┘
                  ↓
        ┌──────────────────────┐
        │     Inoculation      │
        │ (0.5-1.0% Butter Starter) │
        └──────────────────────┘
                  ↓
    ┌──────────────────────────────┐
    │         Incubation           │
    │ (21°C until Acidity reaches 0.6%) │
    └──────────────────────────────┘
                  ↓
          ┌──────────────────┐
          │  Cooling (5°C)   │
          └──────────────────┘
                  ↓
            ┌─────────────┐
            │  Packaging  │
            └─────────────┘
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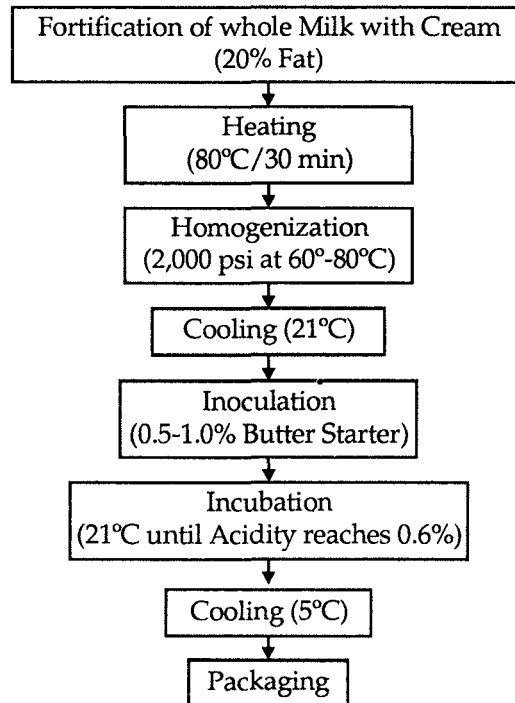
Fig. 11.3 : Flow diagram for the manufacture of sour cream.

11.8 KEFIR

Traditional kefir is an historic and old product from the Caucasus Mountain region in Russia. In the Soviet Union, per capita annual consumption of this product is over 2 kg. The unique feature of kefir is the use of kefir grains as a starter to make a batch of the product. The grains can be re-used several times if proper sanitation is observed in recovering, drying and storing the grains from batch to batch.

(a) **Definition**—*Kefir* is an acidic, mildly alcoholic, distinctly effervescent product made from milk of goat, sheep or cow by fermentation. The fermentation is initiated by the white yellow grains resembling cooked rice, insoluble in water, gelatinous, or irregular size (cauliflower-like) ranging from the size of wheat grains to that of walnuts, distributed on the inner surface of the vat. When added to milk, they swell and turn white, forming a slimy gelly-like product.

Kefir is the most popular cultured product in the Soviet Union and is also produced in small amounts in Czechoslovakia, Poland, Sweden and other East European countries. However, the starters and the manufacturing methods employed in these countries differ considerably and therefore, kefir quality can vary greatly. Traditional kefir was prepared by the inhabitants of Caucasus Mountains in Russia in leather bags made from skin of a goat or oak vats. It was made in these specially designed bags from milk of any of the several animal species after carrying out desirable fermentation. The same method is followed even now in some of these countries. The fermentation is more or less continuous as fresh milk is added to the bag as kefir is removed for use. Ordinarily, the bag is hung out of doors in

the shade during the summer but is kept indoors during the winter for warmth. It is hung near the door so that each person going past can kick or shake the bag to keep the contents mixed. Kefir is not produced in significant proportions in USA and other countries, although, it is made for home use on a small scale. However, in Indian subcontinent, this product is not very popular.

Typically, the starter for kefir is obtained by propagating kefir grains in milk for its application both in home as well as in small and large scale industrial production. A typical kefir grain is characterized by a highly irregular form, the folded or uneven surface, a white or yellow colour and its elastic consistency and acid taste. The diameter of grain may vary from 1-2 to 3-6 mm or more. The active kefir grains float on the milk surface because of their light consistency. Kefir grains are the product of a strong and specific symbiotic association. They always have a definite structure and themselves act as biologically active organisms. When added to milk, they grow, propagate and impart their characteristic properties to next generations resulting into the formation of new grains. However, all efforts to obtain kefir grains from the mixed cultures of the microorganisms present in the original grains were unsuccessful, thereby, suggesting the possible involvement of some undefined factors in the formation of kefir grains which cannot be mimicked under artificial conditions. Scanning electron microscopic studies of kefir grains reveal closely interwoven threads forming the basic structure of grains in which the typical microflora is trapped. The acid in the product is produced by organisms resembling *L. lactis* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* while the alcohol by lactose fermenting yeasts. Other organisms may also be present in the kefir grains but they may not be essential to make kefir.

(b) Microflora of kefir grains—The typical microflora of the kefir grain includes lactic acid and acetic acid bacteria as well as yeasts. Non-lactose fermenting yeasts are found in the deep layers of the kefir grains whereas lactose fermenting yeasts are present in the peripheral layers. The surface microflora of kefir grains comprises mesophilic lactococci, mesophilic and thermophilic lactobacilli and acetic acid bacteria. The microflora of kefir starter which is prepared by growing kefir grains in milk, referred to as "grain starter", consists of several functionally different groups of microorganisms which include the following:

(i) Mesophilic homofermentative Lactococci—These constitute *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris. They* form the largest and most active part of kefir starter microflora, and account for rapid acid development during initial fermentation. However, these are inhibited at high acidity levels.

(ii) Lactobacilli—Several species of lactobacilli are constantly present in kefir grains. However, their number does rarely exceed 10^2-10^3/ml and hence, these microorganisms are of little importance for the quality of the product. The predominating species include the obligate heterofermentative *L. brevis, L. casei* subsp. *rhamnosus* and *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* which are used in kefir preparation. All the strains studied differ from *L. brevis* and other species of heterofermentative lactobacilli. It has therefore, been proposed that they are considered as representatives of the new species *L. kefir.* The taxonomy of mesophilic lactobacilli, *L. casei* subsp. *rhamnosus* should be considered as a separate species with the name *L. rhamnosus* because of its low DNA-DNA homology with reference strain of the species. It has been shown that 20% of the dry weight of the, kefir grains consists of extracellular polysaccharide material produced by *L. brevis* or *L. kefir.* After hydrolysis, the polysaccharide was found to contain atleast 80% glucose and galactose.

(iii) **Mesophilic heterofermentative Lactococci**—These include *Leuconostoc mesenteroides* and *Leuconostoc mesenteroides* subsp. *dextranicum* which impart characteristic taste and aroma to the kefir grains. However, if their growth is excessive, gas formation is observed. *L. dextranicum* strains are activated at elevated temperatures and in the presence of large number of yeasts. By using immunological studies and DNA - DNA homology; it was demonstrated that *L. mesenteroides* is distinct from all other Leuconostocs except *L. dextranicum*. Because of this factor, strains belonging to this species must be considered as a subspecies of *L. mesenteroides*, and should thus, be referred to as *L. mesenteroides* subsp. *dextranicum*.

(iv) **Yeasts**—A number of investigators have found different types of yeasts like *Kluyveromyces marxianus* subsp. *marxianus*, *Torulospora delbrueckii*, *S. cerevisiae*, *Candida kefir*. Yeasts play an important role by promoting symbiosis among the microorganisms, CO_2 formation and development of characteristic taste and aroma. Hence, their excessive growth can cause high levels of gas formation in the product and consequently, packaging problems.

(v) **Acetic acid bacteria (Acetobacter aceti and A. resens)**—They play an important role in maintaining the symbiosis among the kefir grain microflora. Acidification activity is increased when lactococci are cultivated together with acetic acid bacteria. Acetic acid bacteria increases the consistency of the kefir by increasing its viscosity.

(c) **Role of kefir starters**—Kefir starter prepared with grains possesses properties which are very important for the manufacture of the product. This is primarily because the kefir grains provide optimal conditions required for the symbiotic growth of different microorganisms. The starter is neither affected by seasonal fluctuations in milk quality nor by presence of the normally detected quantities of antibiotics or other inhibitors in the milk. The starter is also resistant to phages. Any attempts to replace kefir grains by a starter, composed of pure microorganisms is not effective because the pre-established ratio between microorganisms is altered in the process of transfer and cultivation. A method for a single use kefir starter preparation by means of grinding kefir grains in neutralized kefir and subsequently, mixing with a yeast preparation, was unpractical as the method is rather complicated and cannot compete with the conventional method of kefir starter preparation. If kefir grains are cultivated under optimal conditions, they remain active in the dairy for many years. It is recommended to replace them only in exceptional cases. To make the distribution more convenient, kefir grains are lyophilized in a specially composed medium and after revival in pasteurized milk, their normal characteristics are restored.

Kefir prepared with the grain starter has the typical taste and aroma of kefir and, therefore, it is advisable to manufacture kefir even on an industrial scale, only by using grain starter. Sometimes for kefir preparation, a bulk starter is used, which is prepared by fermenting pasteurized milk with grain starter. This is practised because of lack of equipments for separating kefir grains from the starter. In this case, the starter is prepared in a vessel with a stirrer. Milk is pasteurized with continuous agitation at 95°C for 30 min. and then cooled to 18°C in summer and to 22°C in winter. The milk is inoculated with 2-3% of the starter and carefully agitated. Under normal conditions the fermentation time is 10-12 hrs. The starter ripens in the course of slow cooling to 8°C over 12-24 hrs.

(d) **Composition**—The product usually contains 0.9-1.1 % lactic acid and 0.1-1.0% alcohol alongwith sufficient carbon dioxide to cause effervescence as well as traces of acetaldehyde, diacetyl and acetoin. Of the total nitrogen, 7% is in the form of peptones and 2% as amino acids. There is slight increase in vitamin B_1, B_2 and folic acid content in the final product. Kefir flavour is mildly alcoholic and sour.

(e) Method of manufacture—The steps involved in the manufacture of kefir are shown in the flow diagram (Fig. 11.4).

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                    ┌─────────────────────┐
                    │  Standardized Milk  │
                    └─────────────────────┘
                               ↓
            ┌──────────────────────────────────┐
            │ Heat Exchanger (95°C/5 min)       │
            └──────────────────────────────────┘
                               ↓
                    ┌─────────────────────┐
                    │     Homogenizer     │
                    └─────────────────────┘
                               ↓
                    ┌─────────────────────┐
                    │    Pasteurization   │
                    └─────────────────────┘
                               ↓
                    ┌─────────────────────┐
                    │    Heat Exchanger   │
                    └─────────────────────┘
                               ↓
          ┌──────────────────────┐        ┌────────────────────────┐
          │ Ripening (23°C/20 hr)├───────→│  Kefir Culture (2-3%)  │
          └──────────────────────┘        └────────────────────────┘
                       ↓                              ↓
              ┌─────────────────┐         ┌────────────────────────┐
              │     Stirring    │         │      Kefir Culture     │
              └─────────────────┘         └────────────────────────┘
                       ↓
              ┌─────────────────┐──────────→
              │  Heat Exchanger │
              └─────────────────┘
                       ↓
              ┌─────────────────┐         ┌────────────────────────┐
              │     Filtering   │────────→│      Post-Ripening     │
              └─────────────────┘         └────────────────────────┘
                       ↓                              ↓
              ┌─────────────────┐         ┌────────────────────────┐
              │   Consumption   │         │       Consumption      │
              └─────────────────┘         └────────────────────────┘
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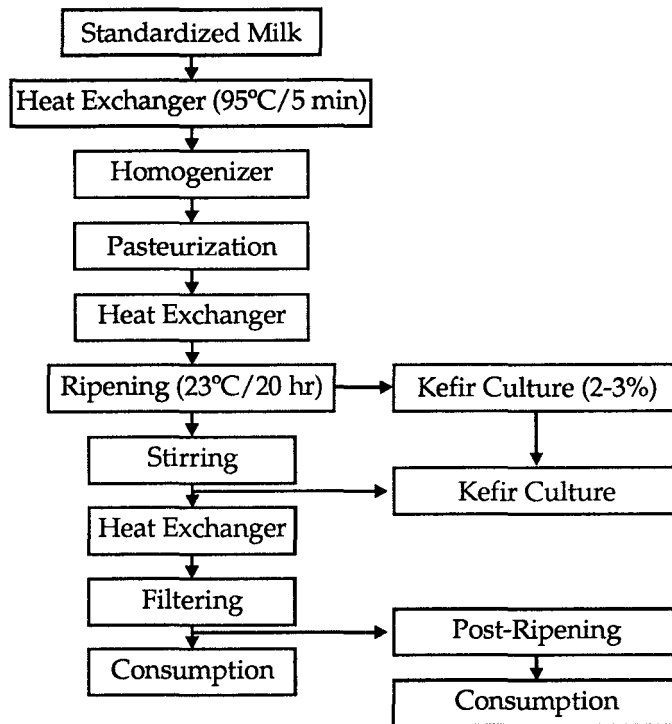
Fig. 11.4 : Flow diagram of manufacture of kefir.

(f) Nutritional value—Kefir is considered to possess the same nutritional value as that of milk. The advantages of the fermentation include acidification of milk, thereby, increasing its storability, prevention of putrefaction and food spoilage organisms. All these features rule out the possibility of milk as the vehicle for transmission of disease organisms. Hence, it can be concluded that kefir forms an attractive sour milk with excellent keeping quality. Since the manufacture of kefir is simple, it could be a low cost method of preserving milk especially in a tropical country like India.

11.9 KUMISS (MILK WINE)

Kumiss is one of the most ancient drinks prepared from mare's milk and is extremely popular throughout Eastern Europe. The name 'Kumiss' was derived from a tribe called Kumanes, who lived along the river Kumane in the Asiatic Steppes. Scythian tribes which roamed from place to place in South East Asia and Middle Asia used to drink mare's milk in the form of Kumiss about 25 centuries ago. The famous Greek Historian Herodot had clearly stated in the 5th century B.C. that Kumiss was a favourite drink of the Scythians who made their prisoners whip mare milk in high wooden vessels. It was consumed as a food as well as a weak alcoholic drink. Marco Polo had also mentioned Kumiss as being a pleasant milk drink. During ancient days, in order to accelerate the fermentation of Kumiss, pieces of horse flesh or tendon or some vegetable matter were added to the mare's milk placed

into bags made from the skin of lamb, presumably to provide microflora needed for fermentation. Mare's milk does not coagulate at the isoelectric point of casein and hence Kumiss is not considered as a curdled product. However, its modem counterpart is based on cow's milk instead of mare's and is now widely consumed in Eastern Europe.

(a) **Definition**—Kumiss is an effervescent acidic, alcoholic fermented, milky white/ greyish liquid made primarily from mare's milk. This product has been described as the greatest of the fermented milks. The organisms responsible for the fermentation in this product are similar to those described for kefir but do not develop masses or grains. Mare's milk or cow's milk or skim milk with added sucrose may be used for preparing this product.

(b) **Composition**—The major end products in Kumiss are 0.7-1.8% (Av. 0.7-0.9%) of lactic acid, 1.3% of ethanol (according to USSR Standard, 0.6-2.5%) and 0.5-0.88% of carbon dioxide. Milk proteins are hydrolyzed in Kumiss but still there is no exact information available concerning the detailed characteristics of Kumiss. Kumiss from mare's milk is of the following composition: -

Lactose%	:	2.3
Fat%	:	1.5
Proteins %	:	2.0
Lactic acid%	:	0.7-0.9
Alcohol%	:	1.2

(c) **Types of Kumiss**—Based on different concentrations of lactic acid and alcohol, three types of Kumiss can be prepared,

i) Low acid and low alcoholic Kumiss
Lactic acid = 0.6%
Alcohol = 0.7%

ii) Medium acidic and medium alcoholic Kumiss
Lactic acid = 0.8%
Alcohol = 1.1-1.7%

iii) High acidic and high alcoholic Kumiss
Lactic acid = 1.0%
Alcohol = 1.7-2.5%

(d) **Microflora of Kumiss**—The microbial content in original Kumiss is extremely variable. The main microflora is represented by *Lactobacillus/Bacterium orienburgii, Lactobacillus delbrueckii* subsp. *bulgaricus, L. acidophilus* and lactose fermenting yeasts, namely, *Torula kumiss* and *Saccharomyces lactis*. It is also possible to find lactococci, coliforms and some spore formingbacilli. Finished Kumiss is of sourish alcoholic flavour and fizzy appearance. Due to high acidity of Kumiss, the growth conditions for contaminating microorganisms are neither favourable at the beginning nor at the end of the manufacturing process.

(e) **Fermentation**—The preparation of milk for Kumiss is the same as for kefir production. The milk is usually inoculated with more than 10% of starter which favours the growth of thermophilic lactobacilli. At the beginning of the fermentation, the product is continuously stirred. The agitation stimulates the proliferation of yeasts.

(f) **Method of manufacture**—The steps involved in the manufacture of Kumiss have been illustrated in the flow diagram. (Fig. 11.5).

```
          ┌─────────────────────────────────────┐
          │   Fresh Mare's Milk or Cow's Milk   │
          └─────────────────────────────────────┘
                            │
          ┌─────────────────────────────────────┐
          │   Heating (90°-92°C for 5 min)      │
          └─────────────────────────────────────┘
                            │
            ┌─────────────────────────────────┐
            │      Cooling (26°-28°C)         │
            └─────────────────────────────────┘
                            │
        ┌─────────────────────────────────────────┐
        │     Inoculation with 30% Starter        │
        └─────────────────────────────────────────┘
                            │
```

Acidity 1.4% in Mare's Milk.	(1 part *L. delbrueckii* subsp. *bulgaricus* at 37°C for 7 hr 2 parts *Torula* sp. at 30°C for 15 hr)

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        ┌─────────────────────────────────────────┐
        │  Incubation (28° till Acidity 0.7-0.8%) │
        └─────────────────────────────────────────┘
                            │
        ┌─────────────────────────────────────────┐
        │        Agitation (every 24 hr)          │
        └─────────────────────────────────────────┘
                            │
            ┌─────────────────────────────────┐
            │        Cooling (20°C)           │
            └─────────────────────────────────┘
                            │
                ┌───────────────────────┐
                │       Kumiss          │
                └───────────────────────┘
                            │
                ┌───────────────────────┐
                │      Packaging        │
                └───────────────────────┘
                            │
          ┌─────────────────────────────────────┐
          │   Storage (4°-5°C for 24 hr)        │
          └─────────────────────────────────────┘
```
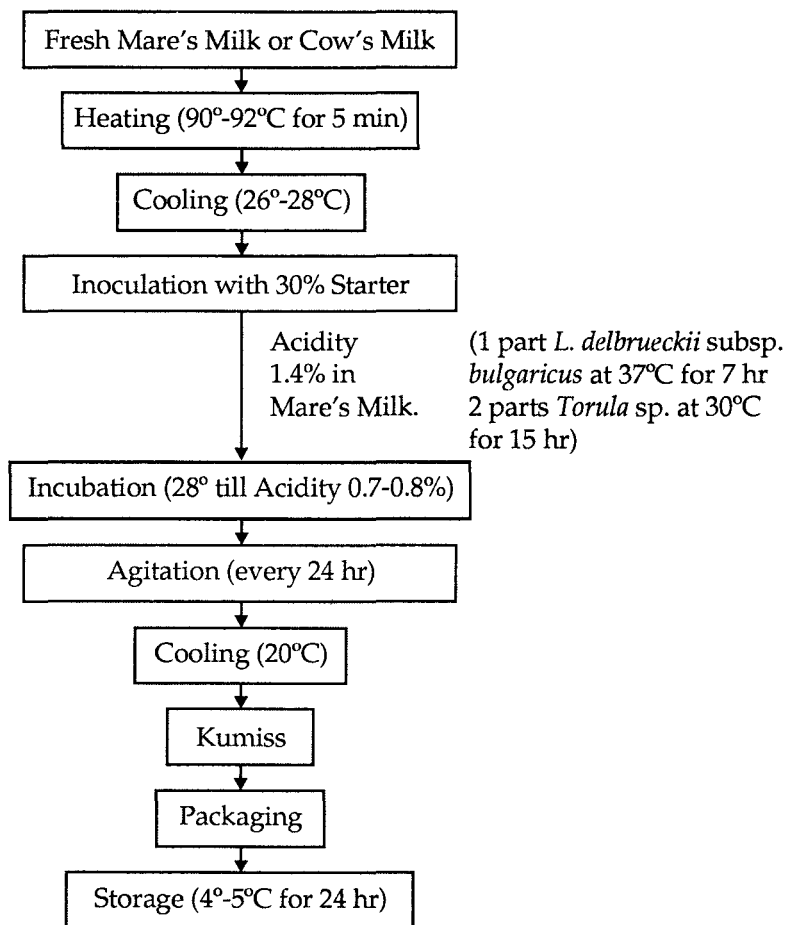
Fig. 11.5 : Flow diagram of manufacture of Kumiss.

In order to produce better quality Kumiss, new starters for Kumiss are proposed. They consist of L. *lactis* subsp. *lactis, L. delbrueckii* subsp. *bulgaricus* and *Saccharomyces cerevisisae* and also included is *Acetobacter acetii* (0.2%) to improve specific aroma. For the use of this starter, new technology was developed and the storage of Kumiss was extended until 14 days. Considering the antibiotic properties of Kumiss it was stated that the original Kumiss as well as freeze-dried preparations possessed high antibiotic activity when strong Kumiss was tested. In comparison with the young Kumiss, the strong preparation showed 51% higher activity. After the completion of processing, a refreshing effervescent drink with a clean lactic flavour can be obtained.

(g) Therapeutic, Nutritive and Dietetic Values of Kumiss—Kumiss is considered to be a refreshing therapeutic drink. In USSR, this preparation is used for the treatment of pulmonary tuberculosis. The significance of therapeutic value of different Kumiss preparations against different diseases has been highlighted. More than 50% Russian Sanitoria recommend Kumiss for treatment of tuberculosis patients and the prescribed dose is 1.5 quartz per day for 2 months.

Compared to cow's milk, mare milk has a different composition. It is richer in albumin, peptones and amino-acids and contains more vitamins C, A, B_1, B_2 and B_{12} alongwith several micro-elements. Kumiss is of liquid consistency in which casein is present in the form of fine flakes which are not felt by the tongue. The nitrogenous substances are easily digestible. Mare's milk differs from cow's milk towards its immunity to *Mycobacterium tuberculosis* and it is known that horses rarely contract tuberculosis. Kumiss made from mare's milk is considered to be a good supplementary remedy for the treatment of tuberculosis. Subsequently, more universal therapeutic effects attributed to Kumiss were discovered and the product improves the nutrition of man and normalised physiological functions. Kumiss is also considered equally effective in the treatment of gastrointestinal diseases, chronic bronchial pneumonia, dry Pleurasy and for people convalescing after the attack of infectious diseases. Extensive research work has been going on to develop a formula and a method of preparing Kumiss from cow's milk which would approximate the chemical composition and properties obtained from mare's milk. Compensation for the immune properties of mare's milk against tuberculosis has been achieved by selecting starter microflora possessing high antibiotic activity against *M. tuberculosis* and Enterobacteria.

11.10 LEBEN AND OTHER RELATED PRODUCTS

Leben is yet another fermented milk in this category which originated in the Middle-East countries. It is a concentrated yoghurt-like or kefir-like product. The condensation is brought about by hanging the fermented curd in a cloth-bag which allows the whey to drain out. In some countries like Turkey, a goat's or sheep's skin bag is used whereas in Egypt instead of bag, an earthenware porous vat through which moisture is removed is used. In some areas, it is rolled in balls and sun-dried to produce a curd mass. The traditional method of making leben is outlined in the flow diagram (Fig. 11.6).
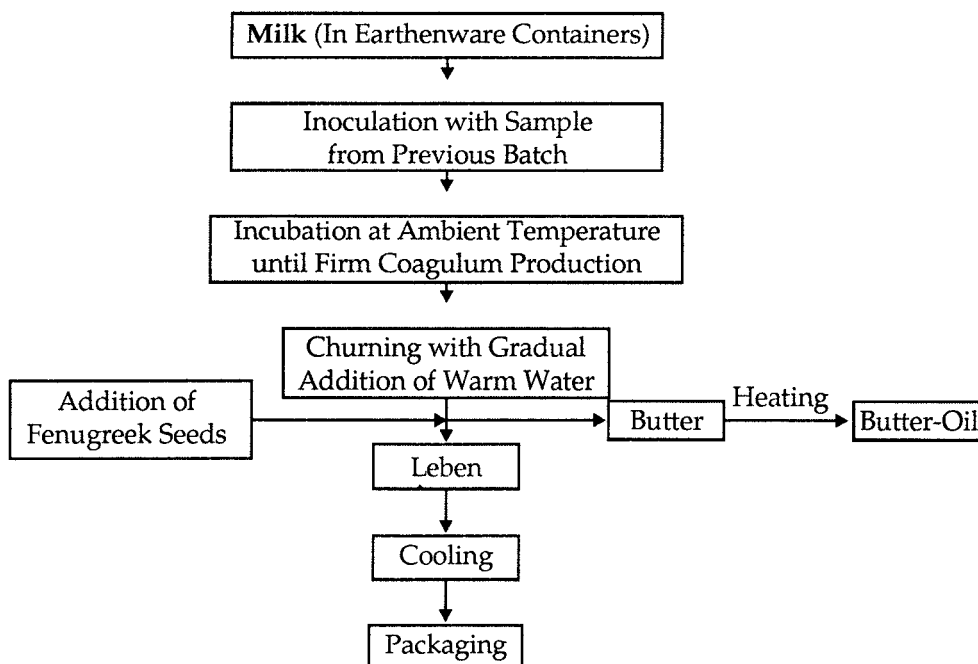

Fig. 11.6 : Flow diagram for manufacture of leben.

There is a mixed microflora consisting of *L. lactis* subsp. *lactis, S. thermophilus, L. delbrueckii* subsp. *bulgaricus* and lactose fermenting yeasts acting in *Leben* production. The related fermented milks are known under different names, e.g. *Labneh* or *Lebneh* from Labanon or other Arabian countries; Tan from Armenia; *Torba, Tubem* from Turkey; *Gioddu* from Italy; *'Matzun'* from USSR.

11.11 SKYR

It is a traditional Icelandic milk product which is somewhat similar to *Quarg* and has the advantage of being rich in protein and low in fat.

(a) **Composition**—The chemical composition of *Skyr* is presented in Table : 11.2.

Table 11.2 : Chemical composition of *Skyr*

Constituent	Level in	
	Skyr	**Skyr whey**
Dry matter (%)	16.72	5.66
Protein (%)	11.54	0.48
Fat (%)	0.19	—
Lactose(%)	4.41	4.40
Lactic acid (%)	1.75	0.94
Ash (%)	0.78	0.75
Thiamine (mg/100 g)	0.77	0.02
Riboflavin (mg/100 g)	0.22	0.09
Calcium (mg/100 g)	111	120
Magnesium (mg/100 g)	9.9	9.8
Phosphorus (mg/100 g)	165	74
Sodium (mg/100 g)	56.5	55.8
Potassium (mg/100 g)	122	156

(b) **Method of Preparation**—The production of *Skyr* originally made on farms has now been industrialized and the dairy cooperatives at some places in the iceland, namely, *Selfoss* have modified the skyr-making process, thereby, improving the quality of this product, shortening the process time and increasing the yield. According to the proceedings of the International Symposium on Nutritional Impact of Food Processing, 1987 'at Reyjkavik, the modern production of *Skyr* involves fermentation of pasteurized skim milk using 0.01-0.1% fresh *Skyr (S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. helveticus)* and rennet. An acid curd is formed at 40°C (pH 4.65), fermented for 16-18 hr at 17°C (pH 4.4) and separated from the whey by centrifugation. The flow diagram for the production of *Skyr* is shown in Fig. 11.7.

Skyr is consumed as such or is mixed with cream and fruit. It also has potential as an ingredient for raw milk products because of its unique texture and high nutritional quality. *Skyr* whey is used as a drink as well as medium for cooking (fish especially) and food preservation. The preservation potentials of *Skyr* whey are considerable as the foods preserved in such way can last for months in a cool place without loss of quality.

```
                    ┌─────────────────────────┐
                    │  Pasteurized Skim Milk  │
                    └─────────────────────────┘
                                    0.01-0.1% Fresh Skyr
                                    (S. thermophilus +
        Rennet ──────────────▶      L. delbrueckii subsp. bulgaricus
                                    + L. helvaticus)
                    ┌─────────────────────────┐
                    │    Fermentation (40°C)  │
                    └─────────────────────────┘

                    ┌─────────────────────────┐
                    │   Acid Curd (pH 4.65)   │
                    └─────────────────────────┘

                    ┌─────────────────────────┐
                    │   Fermentation at 17°C  │
                    │  for 16-18 hr (pH 4.4)  │
                    └─────────────────────────┘

                    ┌─────────────────────────┐
                    │   Skyr Curd in Whey     │
                    └─────────────────────────┘

                    ┌─────────────────────────┐
                    │  Heating to 67°C/15 sec │
                    └─────────────────────────┘

                    ┌─────────────────────────┐
                    │     Cooling (30°C)      │
                    └─────────────────────────┘

            ┌───────────────────────────────────┐
            │  Separation in a Guarg Separator  │
            └───────────────────────────────────┘

        ┌─────────────────────┐     ┌─────────────────────┐
        │        Skyr         │     │        Whey         │
        │ (16-17% Dry Material)│    │  (5% Dry Matter)    │
        └─────────────────────┘     └─────────────────────┘
                                    ┌─────────────────────┐
                                    │   Ultrafiltration   │
                                    │       (50°C)        │
                                    └─────────────────────┘

        ┌─────────────────────┐     ┌─────────────────────┐
        │     Retentate       │     │      Premeate       │
        │ (16-17% Dry Material)│    │                     │
        └─────────────────────┘     └─────────────────────┘
                                    ┌─────────────────────┐
                                    │     Whey Drink      │
                                    │  with Fruit Juices  │
                                    └─────────────────────┘
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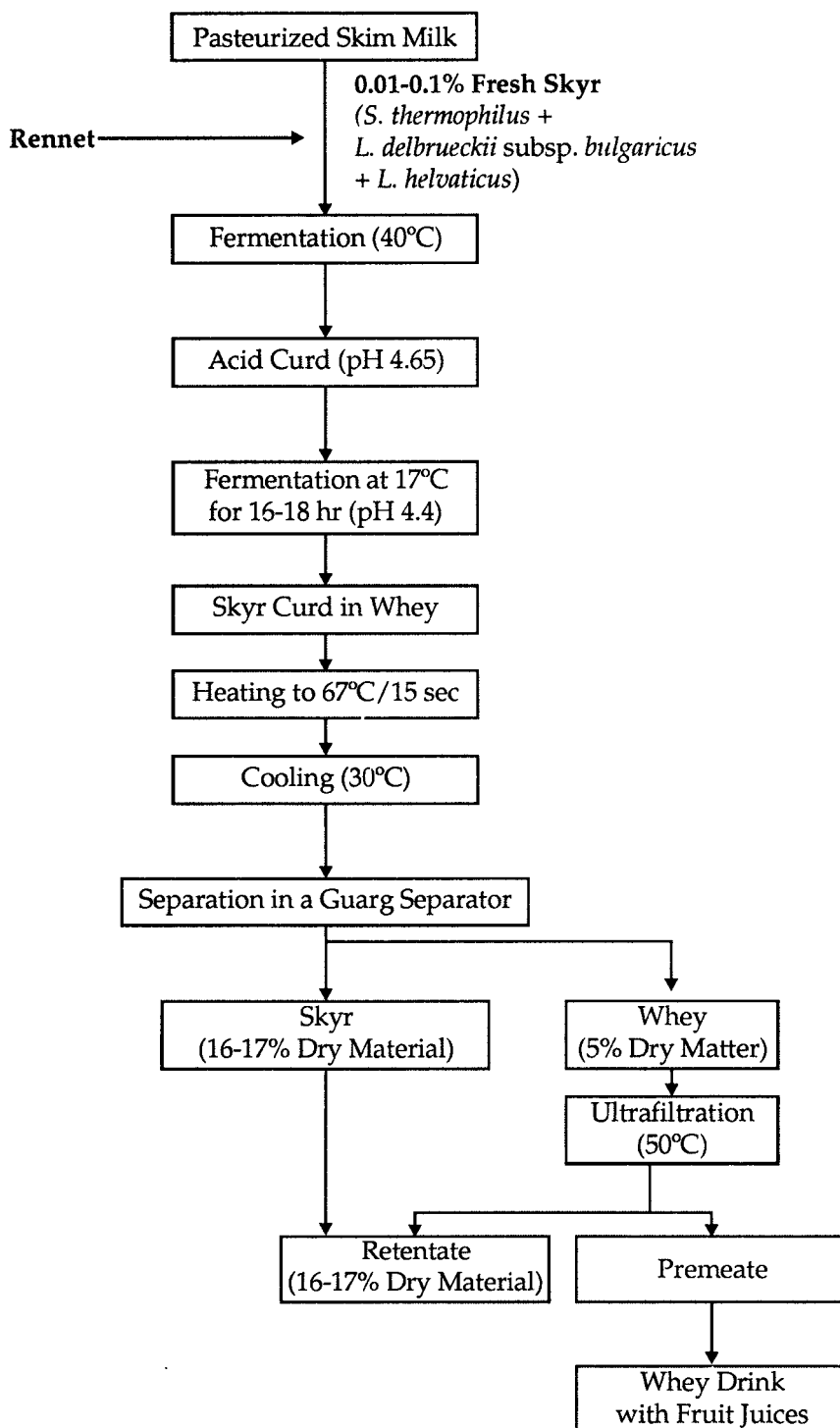
Fig. 11.7 : Flow diagram for the manufacture of *Skyr*.

11.12 TAETTE (SCANDINAVIAN ROPY MILK)

(a) **Definition**—*'Taette' is a moderately ropy and sour milk product of slightly flowing consistency which contains not more than 0.3- 0.5% of alcohol.* This product is extensively used in the Scandinavian countries and in Finland. In the Scandinavian countries, a bulk of taette is produced mainly in the homes by fermenting fresh milk with a strain of *S. lactis* var. *hollandicus*. It is mildly acidic in flavour and is markedly viscous. Although, no recent data on this fermented milk appears to be available, it is reasonable to assume that the product referred to as 'Taet-Majolk' is the same which we now refer to as 'Taette'.
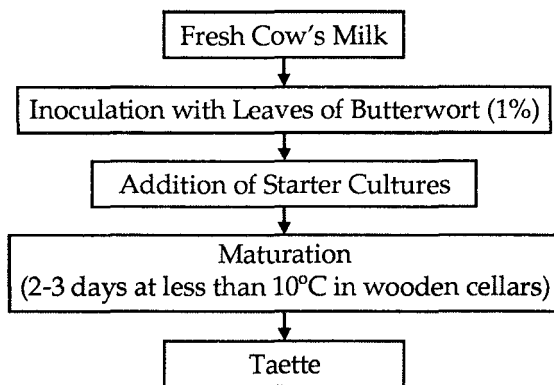
(b) **Composition**—

Lactic acid (%)	:	1.0
Alcohol (%)	:	0.3-0.5
Acetic acid	:	Traces
CO_2	:	Saturation level

The shelf-life of this product and "Taet-Majolk" is approximately 10 months.

(c) **Method of manufacture**—Traditionally, Taette is prepared in wooden cellar which is kept at lower temperature (less than 10°C). As a result of this, longer time is required for the preparation of this product. After removing the final product, milk is again added to the same cellar and the previously left-over product acts as a starter and the process continues.

During the production of Taette, sweet cow's milk is first inoculated with the leaves of butterwort followed by the addition of Taette starter cultures, such as *Saccharomyces major-taette*, *Lactobacillus* taette and *Bacillus acidactislogus*, and *S. lactis* var. *hollandicus*. The flow diagram of method of manufacture is illustrated in Fig. 11.8.

```
              ┌─────────────────────┐
              │  Fresh Cow's Milk   │
              └─────────────────────┘
                         ↓
    ┌──────────────────────────────────────────┐
    │ Inoculation with Leaves of Butterwort (1%) │
    └──────────────────────────────────────────┘
                         ↓
      ┌──────────────────────────────────┐
      │   Addition of Starter Cultures   │
      └──────────────────────────────────┘
                         ↓
    ┌──────────────────────────────────────────┐
    │                Maturation                 │
    │ (2-3 days at less than 10°C in wooden cellars) │
    └──────────────────────────────────────────┘
                         ↓
           ┌──────────────────────┐
           │        Taette        │
           └──────────────────────┘
```

Fig. 11.8 : Flow diagram for manufacture of Taette.

(d) **Microflora of Taette**—The microbiological analysis of Taette suggests a symbiotic association of microorganisms, during the preparation of this product. The microflora of Taette is composed of *S. lactis* var. *hollandicus* (which is instrumental for the production of slimy milk) and some yeasts. The pure culture of *S. lactis* var. *hollandicus* does not curdle milk but does so in combination with yeasts and acidity is considerably increased. The minimum temperature for the growth of this organism is 3°C, whereas optimum is 15°C and the maximum being at 35°C. Besides *L. lactis* subsp. *lactis*, certain lactobacilli may also

be present in Taette. In pure cultures, these lactobacilli are unable to grow at temperature lower than 10°C. However, in association with *S. lactis* var. *hollandicus and* yeasts, they do grow and curdle milk even at lower temperatures, thereby, exhibiting another example of symbiosis. The third type of microorganism in Taette is a yeast of the type *Saccharomyces taette*. In pure culture, this yeast is not capable of fermenting lactose but in combination with lactic acid bacteria, it ferments lactose and produces alcohol upto 2.5%. As a result of these interactions among the different types of microflora, Taette presents a very good example of symbiosis.

11.13 YOGHURT

Whether you spell it yoghurt, yogurt, yogourt, or yoghourt, this food is nutritious fermented milk product consumed in ever-increasing quantities in U.S.A. and Europe. The origin or yoghurt dates back many centuries, although there is no precise record of the date when it was first made. The name according to a legend, yoghurt was first made by the ancient Turkish people in Asia, where they lived as nomads. Another legend tells that an angel brought down the pot that contained the first yoghurt while another claims that the ancient Turks, who were Buddhist, used to offer yoghurts to the angels and stars who protected them. However, others are of the opinion that yoghurt originated from the Balkans. The inhabitants of Thrace used to make soured milk called *Prokish* which subsequently became yoghurt. Later on slaves adopted the procedure of preparation and yoghurt became their traditional food. The nomads who lived in the 5th century along Denube around the Black Sea introduced the procedure of Kumiss preparation for yoghurt production from sheep's milk instead of mare's milk.

The original yoghurt is prepared in Bulgaria from goat's milk (boiled, high solid milk), inoculated at 40-45°C with a portion of previously soured milk. To keep the temperature constant, the pot containing inoculated milk is thoroughly wrapped in furs, placed for 8-10 hr in the oven until a smooth relatively highly viscous, firm cohesive curd with very little wheying-off is formed.

The manufacture of yoghurt and related products in vogue in countries like Turkey is based upon traditional technologies. In principle, there appears to be virtually no difference in manufacture of homemade and factory-made yoghurt worldwide. Modern processes produce milk fermentation under predictable, precise and controllable conditions to yield hygienic fermented dairy products of high nutritional value. All of these products (yoghurt, ayarn, kefir, kumiss, etc.) are made by adding appropriate bacteria and allowing them to grow until the milk is fermented to the desired level. However, in addition to the basic technology dairy companies taken appropriate measures to modify the manufacturing process so as to improve the quality of the final product. These technical steps depend closely on the scale of production of yoghurt. Also new trends are emerging with regard to consumer acceptance of the fermented milks.

Yoghurt is now relished almost everywhere in the world for its characteristic refreshing acid taste, high nutritional value, high digestibility and therapeutic properties. These characteristics are preserved in the fermented food. With the diversification of diet in recent years and their image as medicinal foods, not only a plain yoghurt but also a liquid form (Yoghurt drink) "Ayarn", a frozen form with all kinds of fruits or fruit juices added, a pasteurized or cooked type of yoghurt have appeared in the market of Turkey and other parts of the world.

(a) Definition—*Yoghurt* is defined as a coagulated milk product obtained by lactic acid fermentation of milk with or without additions brought about by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The starter organisms in the final product must be viable and abundant. The classical fermentation process initiated by these organisms impart healthy and nutritious properties to the product. The fermentation brought about by *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus* results in the solid, custard-like texture and an elevated level of developed acidity. Yoghurt as the final product should contain significant numbers of both rods and cocci originally used as the starters. The ability of the cultures to remain viable in the end product is ascribed to their symbiotic growth, metabolic and cellular by-products and a lack of heat treatment to the product. The FAQ/WHO standards No. 11(a) and 11(b) define *yoghurt and flavoured yoghurt respectively as the coagulated milk product obtained by lactic acid fermentation through the action of L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus from milk*. The definition which was used in the first draft of the standard 1 prepared by the secretariat of the milk committee in 1969-70, remained unchanged.

The milk committee considered proposal to delete the reference to specific microorganisms and suggested the inclusion of other species such as *L. acidophilus* but agreed that *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were essential for the production of yoghurt and should remain in the definition but that use of other suitable lactic acid producing cultures should not be excluded. These cultures were listed exceptional additions.

(b) Types of Yoghurt—Following different types of yoghurt preparations are manufactured all-over the world:

(1) Types based on chemical composition—According to FAQ/WHO (1973), yoghurt may be designated on the basis of fat content as full (above 3.0%), medium (3.02-0.5%), or low (0.5% or below) fat yoghurts.

(2) Types based on method of production—Yoghurt can also be classified as (i) set yoghurt, and (ii) fluid yoghurt, based on method of manufacture and physical structure of the coagulum.

(i)   Set yoghurt—It is the product where incubation/fermentation of the milk takes place in the retail container and hence the characteristic coagulum is a continuous semi-solid mass.

(ii)   Stirred yoghurt—It is the type of yoghurt produced after the fermentation has been carried out in bulk and then the coagulum is broken prior to cooling and packaging by stirring.

(iii)   Fluid yoghurt—It can be considered, as stirred yoghurt with low viscosity, e.g. 11% total solids or less.

(3) Types based on flavour—On the basis of flavour, three different types of yoghurt are in vogue.

(i)   Natural or plain yoghurt which is the traditional type with sharp acidic taste.

(ii)   Fruit yoghurt that can be made by the addition of fruits and sweetening agents to the natural yoghurt.

(iii)   Flavoured yoghurt is another type in which fruit ingredient is replaced with synthetic flavouring and colouring compounds.

(4) **Types based on post-incubation processing**—Various types of modified yoghurts can now be prepared based on post-incubation processes as detailed below:

(i) **Pasteurized Yoghurt**—This type of yoghurt can be processed by conventional methods but after fermentation, yoghurt is heat treated to extend its shelf-life.

(ii) **Frozen Yoghurt**—This again is prepared by conventional manner but is subsequently deep-frozen to at least 20°C. It requires higher level of sugar and stabilizers for maintaining the consistency of the coagulum during freezing.

(iii) **Dietetic Yoghurt**—These are low-caloric, low lactose or vitamin/protein fortified yoghurts.

(iv) **Concentrated Yoghurt**—It has total solids of around 24%.

(v) **Dried Yoghurt**—It has total solids between 90-94%.

(c) **Microflora of yoghurt**—There are controversial reports concerning the original microflora of yoghurt. The presence of various physiological groups was reported in earlier investigations but these reports also pointed out that *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were the most predominating and played a vital role in the production of yoghurt.

The microflora of yoghurt can be divided into three groups:

(i) **Essential microflora**—It consists of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus.*

(ii) **Non-essential microflora**—It is represented by homo-fermentative lactic acid strains other than in group (i) and by hetero-fermentative lactic acid bacteria. Some of them may be used beneficially for supplementing the original flora, e.g. *L. acidophilus, Bifidobacterium bifidum, Propionibacterium shermanii* and *L. lactis* subsp. *diacetylactis.*

(iii) **Contaminants**—Yeasts *(Candida mycoderma, C. tropicalis, C. Krusei),* molds, coliforms and other undesirable microorganisms may be present as contaminants.

(d) **Method of manufacture**—The common flow diagram for manufacture of different types of yoghurt is presented in the Fig. 11.9.

(e) **Associative action of starter cultures**—Finished yoghurt is the end product of symbiotic interaction of S. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* growing at temperatures in the range of 40-45°C. A proportion of 1:1 of the rods and cocci forms is considered to be the optimum for flavour and texture production. However 1:5, 1:10 or 2.1:1.2 were also found to be favourable. Associative growth of rod-coccus mixed culture also results in greater acid production than single culture growth. It has been established that numerous amino acids liberated from casein by protease produced by *L. delbrueckii* subsp. *bulgaricus* stimulate the growth of *S. thermophilus.* Similarly, it has been demonstrated that acid production by *L. delbrueckii* subsp. *bulgaricus* is enhanced by formate and carbon dioxide produced by *S. thermophilus,* thus, a two-stage growth pattern by yoghurt bacteria during acidification of milk has been proposed. *S. thermophilus* grows faster during the early part of the incubation due to the stimulatory effect of the amino acids liberated (particularly glutamic acid and proline) by *L. delbrueckii* subsp. *bulgaricus,* thereby removing oxygen and producing acid, CO_2 and formate in milk. After the growth of *S. thermophilus* is slowed by increasing concentrations of lactic acid, the more acid tolerant *L. delbrueckii* subsp. *bulgaricus* increases in numbers due to the stimulatory effect of the compounds generated by lactococci.
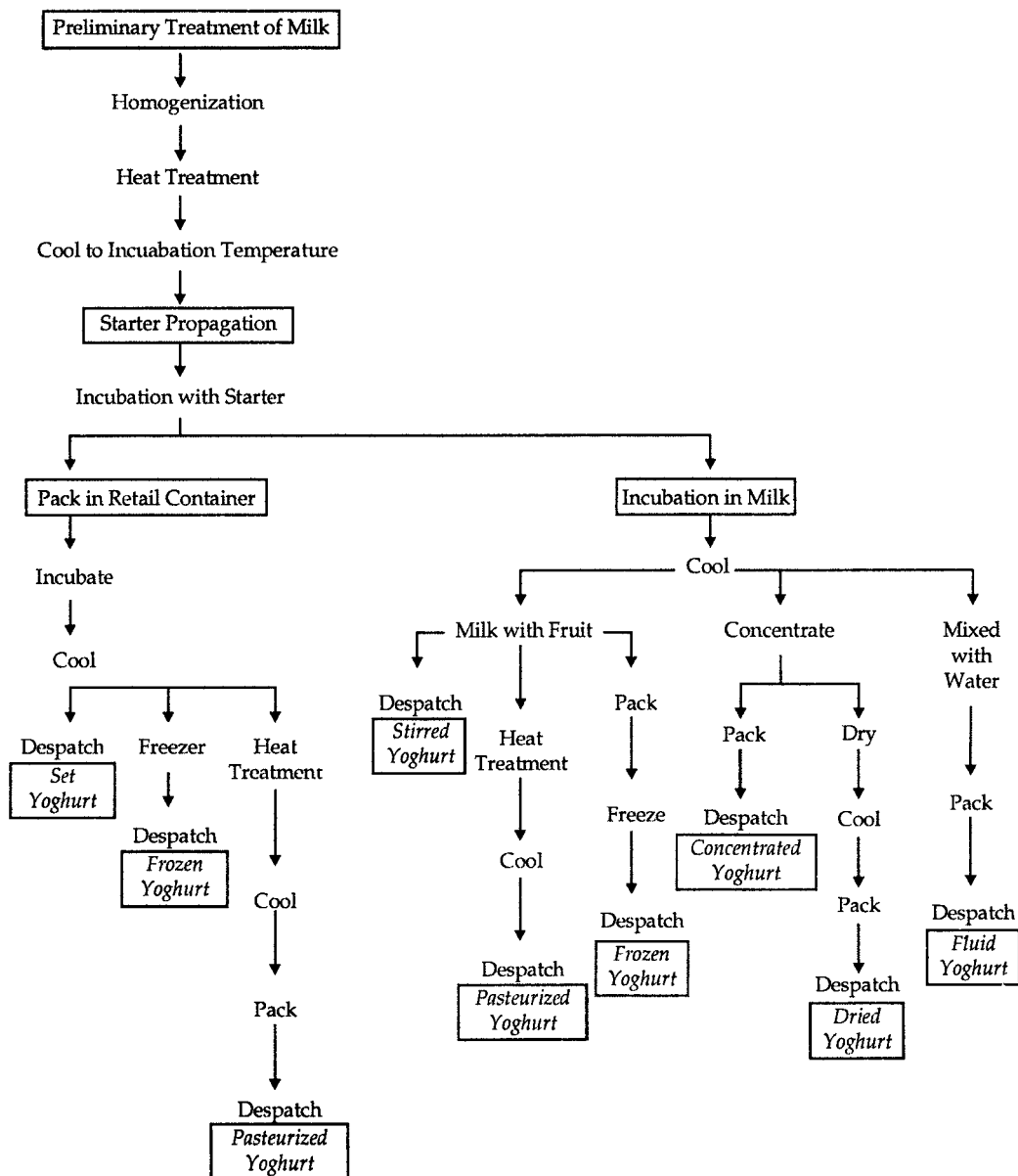
Preliminary Treatment of Milk

↓

Homogenization

↓

Heat Treatment

↓

Cool to Incuabation Temperature

↓

Starter Propagation

↓

Incubation with Starter

Fig. 11.9 : Flow diagram for manufacture of yoghurt.

Yoghurt bacteria particularly *S. thermophilus* exhibit a marked sensitivity towards antibiotics and other inhibitory substances present in milk. Their destruction may also be caused by bacteriophages. Hence, adequate precautions should be taken during propagation of these starter cultures. The two lactic acid bacteria *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* grow in association in milk and for the typical yoghurt starter culture. This growth is considered symbiotic because the rate of acid development is greater when the two bacteria are grown together as compared to the single strains.

The theory of symbiosis was investigated which supported the view that *L. delbrueckii* subsp. *bulgaricus* stimulates *S. thermophilus* by releasing several amino acids (i.e. histidine, leucine, lysine, cystine, valine, etc.), while *S. thermophilus* produces other compounds, such as peptides, purine and pyrimidines, oxaloacetic and fumaric acid, monosodium and disodium orthophosphate. Denatured whey proteins and partially hydrolysed casein has been reported as being stimulatory to yoghurt starter culture. It has also been shown that *S. thermophilus* produces sufficient amount of CO_2 to stimulate the growth of *L. delbrueckii* subsp. *bulgaricus*.

(f) **Therapeutic value of yoghurt**—The therapeutic value of yoghurt was recognized by Metchnikoff as early as in 1900's when he claimed that yoghurt bacteria inhibited the growth of anaerobic sporeformers in the large intestine thus, preventing putrefactive process from taking place in the alimentary tract and thereby prolonging life. By virtue of being a fermented product resulting from acidification of milk by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, yoghurt has now been shown to have an antagonistic effect against a number of pathogenic and spoilage organisms both *in vivo* and *in vitro*. This effect, however, is quite variable. A stronger antagonistic effect of rod-coccus culture to strains of *E. coli*, *Staph. aureus* and *B. subtilis* than each single strain was demonstrated. Culture filtrates of *Streptococcus thermophilus* displayed no antagonistic action against *Staph. aureus*, *E. coli*, *Pseudomonas fragi* and *M. flavus*, while culture filtrates from *L. delbrueckii* subsp. *bulgaricus* and a mixed rod-coccus culture markedly inhibited the growth of test organisms. A study on the survival of coliforms and *Staph. aureus* in yoghurt revealed that these organisms were rapidly inactivated in this product. This further ascertains the tremendous therapeutic value of yoghurt.

Scientific research has documented many nutritional and healthful attributes in live active culture used in the preparation of yoghurts such as antiallergenic effects to milk protein, an increase in calcium bioavailability, the enhancement of bio-availability of other nutrients, the breakdown of lactose to monosaccharides for improved digestibility, the presence of active cultures in the digestive tract, and antibiotic effect against gastrointestinal infections. Additional evidences suggest that yoghurt cultures are important in growth stimulations and longevity, immunostimulant activity, antichloesterolemic activity and also have an anticancer effect.

(g) **Microbiological quality of yoghurt**—In view of the high acidity and antagonistic substances produced as a result of metabolic activity of the two starter cultures, yoghurt can be considered as a hygienically safe product. The potential pathogens like Salmonellae, coliforms etc. as well as spoilage organisms are unable to survive in the natural yoghurt. However, some species of *Staphylococcus* have been found to survive in samples of commercial yoghurt, although there are no records of staphylococcal food-poisoning associated with the consumption of yoghurt. Among the different spoilage organisms, yeasts and molds are chiefly responsible for the deterioration in the quality of yoghurt as these organisms are little affected by low pH. Yeasts and molds can utilize both sucrose and lactose available in the product as energy sources and hence can cause rapid spoilage. Yeasts in particular are a major concern in this respect. Apart from other yeasts, lactose fermenting *Kluyveromyces fragilis* can be potential contaminant. The fruits used in some stirred yoghurts can also be a potent source of these contaminant. The sweetened yoghurt can serve as an ideal medium for the growth and metabolism of *Saccharomyces cerevisiae*. The doming of aluminium foil caps is a common indication of yeast activity. It is suggested that yoghurt should not contain

more than 100 viable cells/ml. Yoghurt with more than 1,000 yeast cells/ml should be considered unsatisfactory. Mold growth in yoghurt, on the other hand, does not constitute a serious problem as commonly occurring molds like *Mucor, Rhizopus, Aspergillus* and *Penicillium* can grow only at the yoghurt/air interface and agitation in stirred yoghurt can suppress their development. Nevertheless, the mold growth can invariably be observed on the surface of the product and its count of 1-10 cfu/ml is normally considered as an indicator of doubtful quality.

The standard methods used for the examination of yoghurt are the standard plate count (SPC), yeast and mold count and coliform count. The routine application of these methods in the quality control labs can tremendously improve the quality of the product. In addition to these, the activity of the starter cultures used in the manufacture of yoghurt also needs to be critically monitored periodically to get product of uniform quality.

(h) **Defects of yoghurt**—Following types of detects are more common to yoghurt.

1. Flavour defects—The most common flavour defect in yoghurt is the absence of typical yoghurt flavour and aroma, mainly because of inadequate acid formation. In spite of the desired balance of cocci and rods in the mother culture, optimum flavour development, occurs only after the acidity reaches about 0.85%. However, beyond 0.95% acidity, a product is obtained which is too sour. Aroma compounds are formed over a considerably wider range of acidity. The absence of typical yoghurt flavour and aroma can also result from the use of strains of *L. delbrueckii* subsp. *bulgaricus* which produce little flavour and aroma substances.

2. Unclean and bitter flavour—These defects in yoghurt result from using poor quality milk or contaminated starter cultures. A few strains of *L. delbrueckii* subsp. *bulgaricus* can cause a bitter taste.

3. Slow acid production—Slow acid production by yoghurt cultures may be due to bacteriophage attack on *S. thermophilus.* Although phage resistant cultures can be obtained, the body of the yoghurt made with these cultures is not as firm as desired.

4. Weak curd formation—Weak curd formation is a serious problem with yoghurt made from milk of normal solids content. A weak curd is most likely to result if the solids content of the milk is low or if a sufficient amount of the milk is from cows which are early in the lactation cycle. Some cows also give milk that inherently forms a weak curd. The firmness of the weak curd in the product can be increased by adding 1-2% of dry milk solids. Addition of small amount of rennet may also increase firmness.

5. Whey separation—This defect is caused by incorrect salt balance in the milk and can be controlled by reducing the heat treatment or by adding small amounts of calcium chloride in the milk.

11.14 INDIAN FERMENTED MILKS

11.14.1 Dahi or Dadhi

(a) **Definition**—According to ISI (1980) now designated as BIS, *dahi* or *dadhi is a product obtained by lactic fermentation of cow or buffalo milk or mixed milk through the action of single or mixed strains of lactic acid bacteria or by lactic fermentation accompanied by alcoholic*

fermentation by yeast. This definition does not include milk coagulated by the addition of acids and milk coagulating enzymes. As per PFA rules(1965), *dahi* or *curd* is a product obtained from pasteurized or boiled milk by souring natural or otherwise, by a harmless lactic acid or other bacterial culture. *Dahi* may contain additional cane sugar (Mishti Doi) and it should have the same per cent of fat. This is an equivalent of yoghurt made from boiled milk after inoculation with mixed starter known as *Jaman (khatta)* which consists of the left-over *dahi* from the previous lot. However, it differs from yoghurt in having less acidity. The different starters used in the manufacture of *dahi* constitutes a complex and heterogeneous flora such as *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. plantarum* and lactose fermenting yeasts. A good quality dahi is of firm and uniform consistency with a sweet aroma and clean acid taste. The surface is smooth and glossy and a cut surface is trim and free from cracks and air bubbles.

(b) History—*Dahi* has been an extremely popular fermented food in the Indian subcontinent which includes India, Pakistan, Bangladesh, Nepal, Sri Lanka etc. This product figures prominently in the ancient Hindu scriptures. The medicinal value of the product has been well documented. *Dahi* may be consumed directly either sweetened or salted and spiced. It is also consumed with other foods such as rice and chapati (wheat loaf). *Dahi* has assumed a special place in the daily dietary intake of Indian population, who prefer to take *dahi* once or twice a day alongwith morning or evening meals.
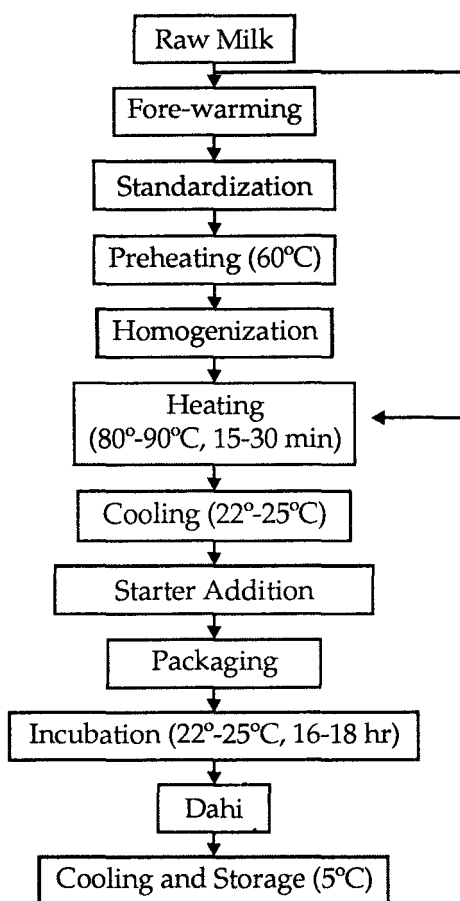
(c) Composition—The composition of *dahi* depends upon the type of milk used and the manufacturing conditions. Following is average composition of *dahi* made from buffalo milk.

<div align="center">

Composition of Dahi

</div>

Water (%)	:	85-88
Fat (%)	:	5-8
Protein (%)	:	3.2-3.4
Lactose (%)	:	4.6-5.2
Ash (%)	:	0.7-0.75
Lactic acid (%)	:	0.5-1.0
Ca (%)	:	0.12-0.14
P (%)	:	0.09-0.11

(d) Method of preparation—The different steps involved in the preparation of *dahi* at household level has been given in the ensuing flow diagram (Fig. 11.10.).

(e) Microflora of dahi—The starters of *khatta (jaman)* used for the preparation of *dahi* is a mixture of lactococci and lactobacilli. The organisms commonly found in the inoculum are *L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis, S. thermophilus; L. acidophilus, L. delbrueckii* subsp. *bulgaricus* and *L. helveticus.* In order to produce a good quality product with a firm and uniform texture, a glossy surface and sweet aroma, the starter inoculum should be free from any contaminating flora. The recommended level of inoculum is 2.0-2.5% with 1-2% acidity to obtain good quality *dahi*. Different workers have recommended different combinations of lactic starters for the preparation of *dahi*, e.g. the National Culture Collection Centre at N.D.R.L., Karnal recommends the use of LF-40 (a mixed culture) as *dahi* culture for the preparation of *dahi* of superior quality.

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│ Cooling (22°-25°C)│
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│Incubation (22°-25°C, 16-18 hr)│
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│    Dahi     │
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│ Cooling and Storage (5°C)  │
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Fig. 11.10 : Flow diagram for manufacture of *dahi* by standardized method.

(f) **Special attributes of dahi**—In Indian system of medicine (Ayurveda), the use of *dahi* has been strongly advocated for curing ailments like dyspepsia, dysentery and other gastrointestinal disorders. The product is also believed to improve appetite, vitality and increases digestibility. Some of the beneficial effects of *dahi* are attributed to the antibacterial components formed during the fermentation and the low pH that prevents the growth of putrefactive and other undesirable organisms including potential pathogens.

(g) **Nutritional value of dahi**—As a result of metabolic activity of *dahi* starters, quantitative nutritive changes occur in milk during dahi-making process. There are appreciable changes in mineral and vitamin contents of *dahi* depending on the type of organism used for fermentation. A mixed culture of *L. delbrueckii* subsp. *bulgaricus* and *Leuco. cremoris* decreased thiamine, riboflavin and nicotinic acid in milk during *dahi* fermentation. However, single culture of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* raised the thiamine concentration from 2 to 20% over that of milk. Similarly, riboflavin content of *dahi* made from whole buffalo milk is almost fourfold that of skim milk *dahi*. The mineral and vitamin contents of *dahi* could be compared with those of milk, as has been shown in Table 11.3.

Table 11.3 : Mineral and vitamin content of *dahi*

constituent	Milk (per 100 g of the product)	Dahi (per 100 g of the product)
Mineral matter (g)	0.8	0.8
Calcium (mg)	149.0	149.0
Phosphorus (mg)	96.0	93.0
Vitamin A (I.U.)	118.0	102.0
Thiamine (µg)	55.0	49.0
Riboflavin	167.0	157.0
Nicotinic acid (µg)	96.0	86.0
Biotin (µg)	29.0	3.2
Pantothenic acid (µg)	202.0	183.0
Folic acid (µg)	161.0	178.0
Vitamine B_{12} (µg)	0.15	—
Ascoribic acid (mg)	1.4	1.3

11.14.2 Srikhand

(a) **Definition**—*Srikhand is sweetened-dewatered dahi.* This product is extremely popular in Western and some parts of Southern India. It has a distinctive rich flavour and fairly long shelf-life. This product too had its origin in ancient India just about the time when *dahi* was introduced.

(b) **Composition**—Although, there are slight variations in the method of preparation of *srikhand*, and, the approximate average composition of this product is given below.

Composition of Srikhand

Moisture(%)	:	34.48-35.66
Fat (%)		1.93-5.6
Protein (%)	:	5.33-6.13
Reducing sugar (%)	:	1.56-2.18
Sucrose (%)	:	55.55-58.67

(c) **Preparation**—During the preparation of *srikhand, dahi* is suspended in a muslin cloth until all the free water has drained off. The semisolid mass (called *chakka*) is then whisked with sugar through a fine cloth, coloured and scented with saffron or rose water and flavoured with cardamom, if desired. In order to further extend the shelf-life of *srikhand*, a preparation known as 'Srikhand wadi', which is essentially a desiccated *srikhand*, is made. The dewatered *dahi* is mixed with an equal amount of sugar by weight and dried in an open pan at low heat. When the mass begins to harden, it is tested for stickiness. The non-sticky product is flavoured and coloured. Powdered sugar is then further added as desired. The product is mixed, rolled cut into shapes and packed like biscuits.

(d) **Microflora of Srikhand**—The microflora of *srikhand* is more or less like that of *dahi*. It includes *dahi* starter and the contaminants. Yeasts may be the most predominant

contaminants and the low quality sugar may be the possible source of these organisms. Apart from these types, potential pathogens may also gain access into the product due to unhygienic practices used in the preparation of *srikhand,* although no food poisoning outbreaks have been reported so far. The nutritional and therapeutic attributes of *srikhand* have not been studied extensively but this product is expected to be comparable to *dahi* in these respects.

11.14.3 Lassi

Lassi is a by-product obtained during the preparation of country butter from *dahi* by indigenous methods. This product is used with or without sugar or salt depending upon the consumer's preference. It is very popular in the countryside and according to a rough estimate, approximately 2,114 million kg of *lassi* is produced in India, annually.

(a) **Composition**—The composition of *lassi* varies considerably since there is no standard method available for the preparation of this product. The factors affecting the composition of lassi are the type of milk used, extent of dilution during churning and efficiency of fat removal. The average composition of *lassi,* is presented below.

<div align="center">Composition of Lassi</div>

Water (%)	:	96.2
Fat (%)	:	0.80
Protein (%)	:	1.29
Lactose (%)	:	1.2
Lactic acid (%)	:	0.44
Ash (%)	:	0.40
Ca (%)	:	0.60
P (%)	:	0.04

(b) **Method of preparation**—*Lassi* can be prepared by churning *dahi* with frequent addition of water until butter granules are formed. The product obtained by manual removal of butter granules is called *lassi* or *chhachh. Lassi* could be preserved for more than six days at 37°C by addition of 0.003 to 0.35% sodium metabisulphite The characteristic sulphur flavour imparted to the product during storage could be masked by the addition of 0.07 to 0.09% crushed green ginger and 0.5-0.7% salt.

(c) **Microflora of Lassi**—The microflora of *lassi* and its microbiological quality are almost comparable to those *of dahi* from which it is derived.

11.15 LACTIC BEVERAGES

Apart from the traditional fermented products, lactic starters have also now stepped into the manufacture of non-conventional cultured milk or whey drinks in a big way, particularly in the western world. These lactic beverages continue to attract the dairy technologists for making these drinks more and more palatable to the consumers and also for providing an economic outlet for milk. During the last few decades, the number of lactic beverages, both milk and whey based have increased considerably. Some of the most common and popular lactic drinks which have recently come into the market are discussed below:

11.15.1 Milk Based Beverages

(a) Drinking yoghurt—Drinking yoghurts are among the potentially most exciting dairy products. A drinking yoghurt with a clean shelf-life of 6 months at room temperature is prepared from skim milk yoghurt by adding fruit juice and sugar. The product which contains 7.6% SNF, 8% sugar and 0.35% pectin is homogenized and pasteurized at a temperature of 73°C for 20 sec, before being cooled and packaged aseptically.

(b) Acidophilus drink—An acidophilus drink using Lactobacillus acidophilus as a starter culture has been developed, recently from recombined milk. The product has a clean acceptable flavour that is similar in many respects to Leben and is liked not only by the western consumers but is equally popular in North Africa.

(c) Alcoholic milk beverages—There also appears to be growing interest worldwide in the alcoholic dairy beverages. One such product has been developed in Japan. It is prepared from skim milk (10%) by culturing with L. lactis subsp. lactis, L. delbrueckii subsp. bulgaricus for 12 hr at 30°C, followed by heating at 75-80°C, addition of 10% sucrose and fermentation with Saccharomyces at 30°C. The product contains 8% ethanol and attains a pH of 4.3 after 10 days. Similar types of other drinks have also been developed in other countries.

11.15.2 Whey Based Beverages

Whey is a by-product of cheese manufacture. Over a long period of time, many attempts have been made to make whey suitable for human consumption. Whey as such by its taste and consistency, is unsuitable for human consumption. It has to be therefore, modified appropriately before it can be utilized in human food. New developments in the membrane technology and reverse osmosis has the possibility for the revaluation of whey and underlines the change of image which whey has undergone during the last two decades. In some countries like the Netherlands, most of the cheese whey is used for the manufacture of whey powder, lactose and low lactose whey products. As often seen in low cost by-products, whey is used as a basic component for fermentation process in the production of, for instance, alcohol or the production of microbial protein.

Whey is of two types i.e. acid whey and rennet whey. When milk is acidified to pH 4.6, most of the caseins are precipitated except the protease peptone fraction. After separation of these precipitated fractions of caseins, the remaining liquid fraction is called whey, which contains several proteins. Most of these are globular proteins and are less soluble. Rennet whey contains well defined part of k-casein and caseino-micropeptidase which results due to the action of chymosin. Though the protein contents of both the types of whey is the same (0.6%), they vary in lactose and calcium content. Besides protein, whey contains a large variety of compounds of lower molecular weight. Most of these dry solids are lactose (46g/kg whey). A wide range of minerals and essential trace elements and vitamins are available in whey. Some amount of vitamin A, B_6 and biotin, organic acid like citric acid and formic acid are also found in whey which helps in aroma production and growth of microorganisms during the fermentation of whey.

Types of whey beverages—Beverages based on whey or partially based on whey are numerous. From the technological point of view, whey drinks can be made from natural whey or whey permeate or refined whey or ultrafiltered whey (retentate) or whey concentrate. Basically these products can be classified from the technological point of view into three main groups:

(a)   Natural (sweet) beverages

(b)   Acid beverages

(c)   Non-alcoholic whey beverages

However, from consumption point of view, whey beverages can be classified into other groups such as: -

Pleasurable or agreeable

Healthy   —   for special occasions (sports)

              —   for diets

              —   for stimulation of metabolic activity

Nutritional   —   immitation of milk

(a) Natural (sweet) Beverages—Natural whey beverage preparation do not involve fermentation process in the course of their manufacture. The most well known common whey beverages available in the market are chocolate based drinks. A chocolate drink based on whey has to be stabilized in order to prevent sedimentation of cocoa particles. In the manufacture of chocolate milk, the thickening agent, k--carrageenan is very useful.

(b) Acid Beverages—Whey can be acidified directly by the addition of, for instance, citric acid or lactic acid or bybacterial activities. Bacteriological acidification is more complicated but provides more special characteristics to the beverages and no additives has to be declared. For ease of handling, the use of non-gas forming starters is recommended for this purpose. In general, mesophilic starter bacteria (Lactobacillus) are used for whey fermentation. Due to low pH value of acid-whey drinks, a relatively mild temperature treatment is sufficient to guarantee a long shelf life of the product.

These beverages are often categorised as soft drinks. Whey is genuine thirst quencher unlike most of other soft drinks. Whey drinks are light and refreshing but less acidic than fruit juices. They possess favourable health and nutritional aspects. Acid whey beverages are usually intended to compete with other soft drinks as there are good potential margins expected from such beverages.

(c) Non-alcoholic Whey Beverages—Non-alcoholic whey beverages also continue to feature widely in the literature. One of the most successful commercial whey beverages of recent years is 'Rivella' developed in Switzerland. Another interesting whey beverage is Nature's Wonder, a 100% whey based beverage developed by a Swedish dairy company. In India also, L. acidophilus based whey beverage, popularly known as 'Acido-Whey' has been developed at National Dairy Research Institute, which is becoming increasingly popular being a refreshing cold drink with pleasant taste and aroma characteristics. A part from this, the drink has been claimed to possess strong therapeutic potentials against gastric disorders. The flow diagram of the Acido-Whey preparation is presented in the Fig. 11.11.
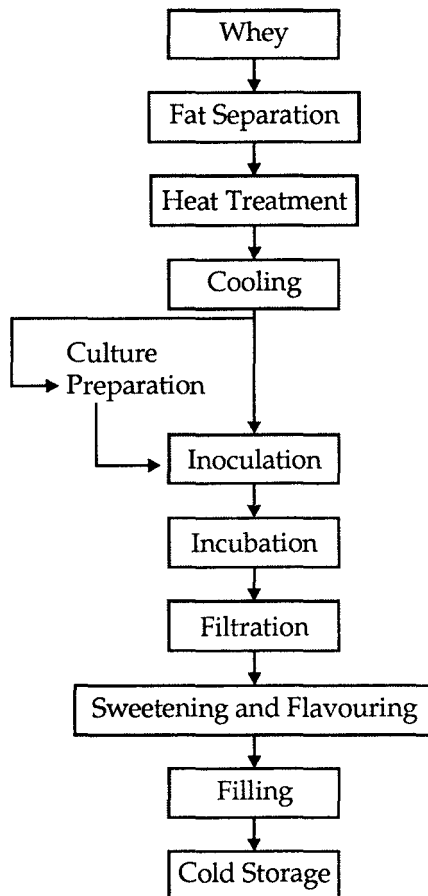
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                  │  Fat Separation  │
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Fig. 11.11 : Flow diagram for the manufacture of "Acido-Whey".

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12

MICROBIOLOGY OF CHEESE

12.1 INTRODUCTION

Cheese is a valuable means of conserving many of the nutrients in milk. In many people, it evokes a similar response to wine, playing an indispensable part in the gastronome's diet and prompting Brillat-Savarin (1755-1826) to coin the rather discomforting aphorism that "Dessert without cheese is like a pretty woman with only one eye." Despite this, the attraction of a well-ripened cheese eludes many people and it is sometimes hard to understand how something that can smell distinctly pedal can yield such wonderful flavours. This paradox was encapsulated by a poet, Leon-Paul Fargue, who described camembert cheese as "the feet of God".

Today cheese making is a major industry worldwide, producing something approaching 15 million tonnes per annum. Much is still practiced on a relatively small scale and accounts for the rich diversity of cheeses still available. There are several hundred known varieties of cheese. The USDA Handbook publication has over 2000 names indexed. Many of these cheeses are named after the town, community, or region where they are made and though known by different names, many have very similar characteristics. However, there are about 400 established varieties and 18 distinct types of cheese. Thus, the spectrum of cheese encompasses not only varieties like Cheddar, Cottage, Gouda, Mozzarella, Emmental (Swiss), Edam, Roquefort, Comembert but also Paneer and Channa. Large-scale industrialized production is increasingly important, however, and is dominated by one variety, Cheddar, which is now produced throughout the world (including India), far removed from the small town in Somerset where it originated.

Modern cheese making is controlled and has been refined through strict adherence to manufacturing guidelines and careful selection of specific lactic acid bacteria and ripening microorganisms. This chapter discusses microbiological aspects of some major types of cheese.

12.2 DEFINITION AND TYPES OF CHEESES

In simple terms, cheese is *"the curd of milk separated from the whey and pressed into solid mass."* It is defined as *"a product made from the curd obtained from milk by coagulating the casein with the help of rennet or similar enzymes in the presence of lactic acid produced by added or adventitious microorganisms, from which part of the moisture has been removed by cutting, cooking and/ or pressing, which has been shaped in and then ripened by holding it for sometime at suitable temperatures and humidities."*

According to the description of hard cheese in PFA rules, cheese (hard) means the product obtained by draining after coagulation of milk with a harmless milk coagulating agent under the influence of harmless bacterial cultures. It shall not contain any ingredients not found in milk, except coagulating agent, sodium chloride, calcium chloride (anhydrous salt) not exceeding 0.02% by weight, annatto or carotene colour and may contain certain emulsifiers and/or stabilizers, viz., citric acid, sodium citrate or sodium salts of orthophosphoric acid and polyphosphoric acid, not exceeding 0.2% by weight; the wax used for covering the outer surface should not contain anything harmful to the health. Hard cheese should contain not more than 43% moisture and not less than 42% milk fat of the dry matter. Hard cheese may contain 0.1% of sorbic acid or its sodium, potassium or calcium salt; or 0.1% of nisin.

Types of Cheeses

The classification of cheeses is based on a number of factors like raw material, type of consistency, appearance (interior and exterior), fat content, moisture content and ripening methods. However, the most commonly used criteria are the moisture content of the finished product and the mode of ripening.

    (i)   Types based on moisture content:

        (a)   Very hard (maximum 34% moisture)

        (b)   Hard (maximum 39% moisture)

        (c)   Semi-hard/ Semi-soft (39-50% moisture)

        (d)   Soft (50-80% moisture)

    (ii) Types based on mode of ripening:

        (a)   **Bacteria ripened**—Ripening is brought about by different bacteria like lactococci, lactobacilli, pediococci, leuconostocs, propionibaeteria and brevibacteria etc.

        (b)   **Mold ripened**—Ripening is brought about by mold species like *Penicillium.*

        (c)   **Unripened**—Ripening is not done.

The inter-relationship between the above two types of classification is presented in the following Fig. 12.1.
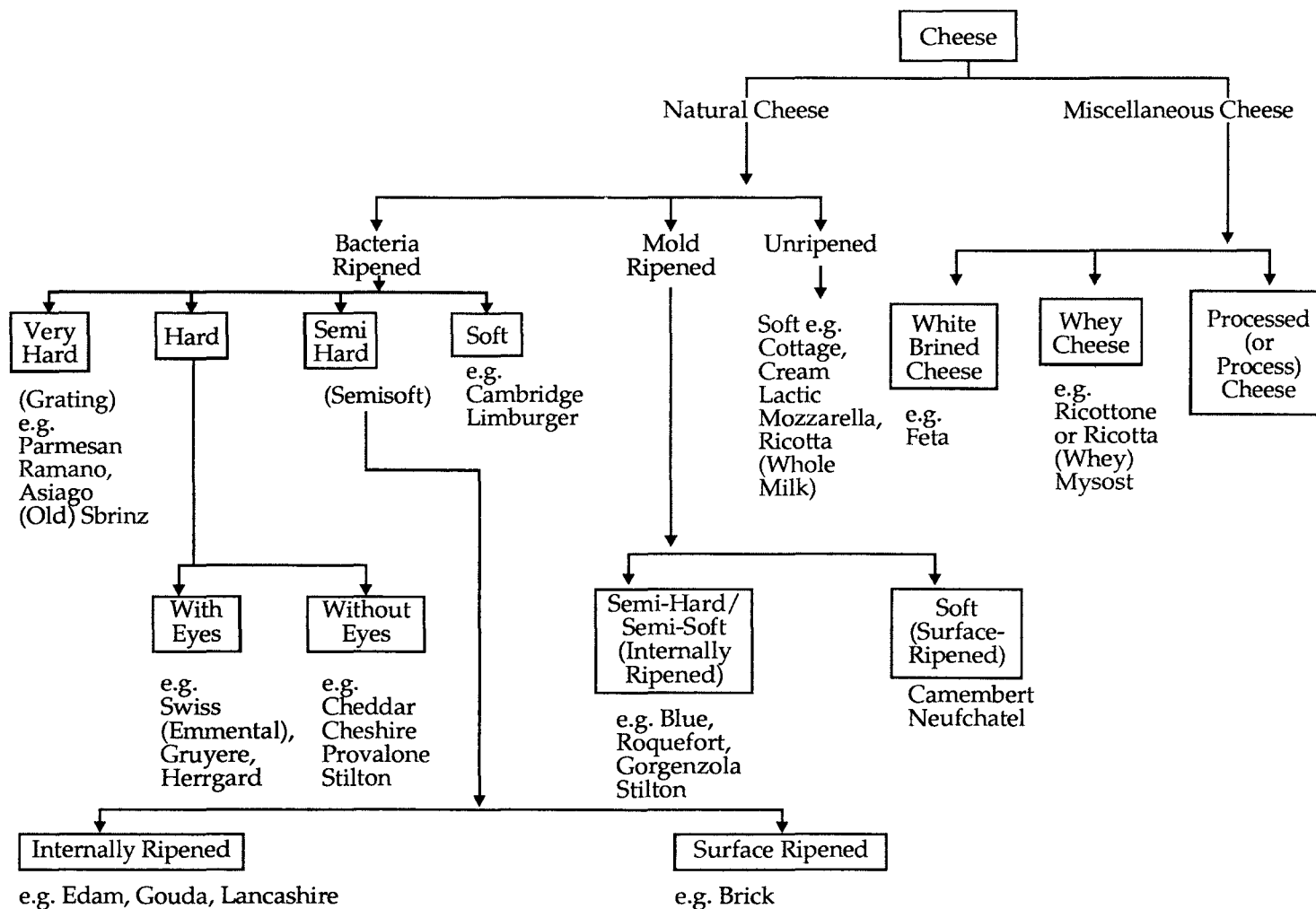
Fig. 12.1 : Inter-relationship between different classes of cheese.

12.3 COMPOSITION

Cheese constituents, viz., fat, protein, moisture, minerals and vitamins differ greatly with the variety of the product. Table 12.1 gives the composition of different types of cheeses.

Table 12.1 : Composition of different types of cheese

Types of cheese	Moisture (%)	Fat (%)	Protein	Calcium (%)	Vitamins			Energy content (Kcal/1 00g)
					Vitamin A (µg/100g)	Thiamin (µg/100g)	Riboflavin (µg/100g)	
Hard (Cheddar)	35.0	33.0	26.0	0.83	380	50	0.50	400
Semi-hard (Edam)	43.0	24.0	26.0	0.76	250	60	0.35	320
Blue-veined (Roquefort)	40.0	31.0	21.0	0.32	300	30	0.70	360
Soft (Camembert)	51.0	23.0	19.0	0.38	240	50	0.45	280
Unripened (Cottage)	79.0	0.4	16.9	0.09	3	30	0.28	82

Source : Porter, J.W.G. (1975), "Milk and Dairy Foods", Oxford University Press, Oxford

Average analyses of some commercial varieties of cheese are mentioned in Table 12.2

Table 12.2 : Average analysis of some varieties of cheese[1]

Variety	Authority	Water, per cent	Fat, per cent	Pro-teines, amides, etc., per cent	Milk sugar, lactic acid, etc. per cent	Total ash, per cent
1	2	3	4	5	6	7
Brick	Bureau of Chemistry	42.47	30.66	21.05	...	2.98
Brie:						
Imported	Bureau of Chemistry	52.53	22.44	20.94	...	4.81
American	Bureau of Chemistry	52.10	24.72	19.60	...	4.06
Caciocavallo	Bureau of Chemistry	34.95	21.98	34.33	...	6.96
Camembert, imported	Bureau of Chemistry	47.88	26.32	22.21	...	4.11

... contd.

1	2	3	4	5	6	7
Cheddar skim	Bureau of Chemistry	57.04	4.88	32.09	...	3.76
Cream-French, Demisel	Bureau of Chemistry	42.74	39.88	14.40	...	1.88
Edam (American)	Bureau of Chemistry	38.07	22.65	30.89	...	6.19
Emmenthaler	Lindt	33.00	30.50	30.44	...	4.17
Gorgonzola	Musso	37.30	34.67	25.16	1.62	3.82
Gouda	Cribb	54.79	9.02	25.94	...	5.52
Gruyere	Balland	29.99	28.19	33.03	4.82	3.96
Limburger:						
American	Arnold	35.64	29.82	28.53	...	5.98
Imported	Bureau of Chemistry	54.79	19.61	21.27	...	5.17
Monster (American)	Bureau of Chemistry	40.60	31.00	22.20	...	4.63
Neufchatel	Bureau of Chemistry	52.05	23.51	19.33	...	4.97
American	Bureau of Chemistry	59.22	18.17	21.30	...	2.43
Parmesan:						
Formaggio	Bureau of Chemistry	16.95	22.71	49.39	...	7.59
Reggiano	Bureau of Chemistry	29.63	27.29	34.84	...	4.76
Pecorino	Sartori	29.80	30.51	33.51	...	6.24
Romano	Bureau of Chemistry	29.56	27.69	31.20	...	8.66
Pineapple	Johnson	24.07	38.12	29.35	2.49	5.69
Port du Salut	Bureau of Chemistry	50.10	25.17	21.18	...	2.91
Roquefort	Currie	38.69	32.31	21.39	...	6.14
Stilton	Bureau of Chemistry	33.57	31.19	28.96	...	3.00
Swiss:						
American	Bureau of Chemistry	34.28	32.60	27.55	...	4.16
Imported	Bureau of Chemistry	33.91	30.61	29.22	...	4.16

[1] Data tabulated from U.S. Dept. Agr., Bull, 608 (Revised).

12.4 BASIC PROCESSES INVOLVED IN CHEESE PRODUCTION

Cheese production involves the following three main steps—

(A) Coagulum formation,

(B) Separation of curd from whey, and

(C) Ripening of cheese.

(A) Coagulum Formation—Milk coagulation occurs due to two distinct activities—

(1) Inoculation with bacterial cultures, e.g. *Streptococcus lactis* or *S. cremoris* (for incubation at 31°C), or *S. thermophilus* combined with *Lactobacillus lactis, L. bulgaricus* or *L. helveticus* (for incubation at 50°C), results in lactose degradation to produce lactic acid, which lowers the pH to about 4.6.

(2) Incubation with rennet cleaves k-casein into para-k-casein and caseino macropeptide. This cleavage occurs at a specific peptide bond between phenylalanine at position 105 and methionine at position 106, and leads to coagulation of α-and β-caseins and the k-casein hydrolysis products.

k-casein stablizes the colloidal nature of milk. The N-terminal region of k-casein is hydrophobic and it associates with the lipophilic regions of α- and β-caseins, which are insoluble. The C-terminal region of k-casein is hydrophilic, and associates with water molecules. Thus an intact k-casein molecule keeps the insoluble α- and β-casein in suspension and prevents their coagulation. Hydrolysis of k-casein by rennet separates its hydrophobic and hydrophilic regions and, thereby, eliminates its protective influence. As a result, the α and β-caseins plus the k-casein hydrolysis products precipitate and form the coagulum. Calcium is essential for coagulation, and the process is very temperature dependent.

Traditionally, rennet obtained from the fourth stomach of unweaned calves has been used. But at present, rennet from microbial sources is used extensively, and is responsible for about 70% of US and 30% of the worldwide cheese productions. The rennet obtained from *Mucor miehei* is relatively more thermostable and hence remains active during ripening, which often produces bitter off-flavours. Therefore, it is treated with oxidising agents like H_2O_2, which convert the methionine residues to their sulphoxides. This reduces the temperature tolerance of the enzyme by 10°C and makes *M. miehei* rennet more comparable to calf rennet. Attempts to clone calf chymosin gene in *E. coli* and *Sacch. cerevisiae* have been successful, but active rennin is secreted only by the yeast cells.

(B) Separation of Curd—The coagulum is heated to 37°C and cooled. This eliminates the remaining rennet activity and separates, to some extent, the watery fluid called *whey*. The curd is separated from whey, salted, and mixed with proteases and/or lipases; alternatively, bricks of cheese may be inoculated with specific strains of fungi, e.g. *Penicillium roquefortii, P. camembertii,* etc. The bricks are pressed to remove excess moisture to enable proper ripening.

(C) Ripening—Ripening procedures will vary with the type of cheese to be produced. The cheese bricks are inoculated with specific strains of fungi (*P. roquefortii* and *P. camembertii*) for the development of appropriate flavours through protease and lipase activities. Alternatively, proteases and lipases may be used for this purpose. Proteases from *B. amyloliquefaciens* are used to enhance flavour in cheddar cheese.

Proteases hydrolyse proteins to produce peptides of variable sizes. Peptides having terminal acidic amino acid residues produce meaty, appetising flavours. But hydrophobic amino acid residues located nonterminally produce bitter flavours; the flavours are the strongest in medium-sized peptides, are absent in longer peptides, and they decrease with a decrease in the peptide size. Therefore, the kind and the degree of flavour in cheese can be controlled by regulating protein hydrolysis.

The stronger flavours of Italian cheeses are produced by a modest lipid hydrolysis, which increases the amount of free butyric acid. Lipolysis is brought about by lipase from *Mucor miehei* or *Aspergillus niger*; the lipase is added to the milk at 30 U/l before addition of rennet.

Thus, cheese production presents examples of following—

(1)   Use of microorganisms for processing of food to enhance flavour, texture etc.

(2)   Use of enzymes in food processing;

(3)   Enzyme (rennet) modification (by chemical reaction) to suit specific needs, and

(4)   Use of recombinant DNA technology to produce enzymes for food processing (cloning to chymosin gene).

Various steps involved in cheese production, alongwith biochemical activities involved, are summarized in Fig. 12.2.
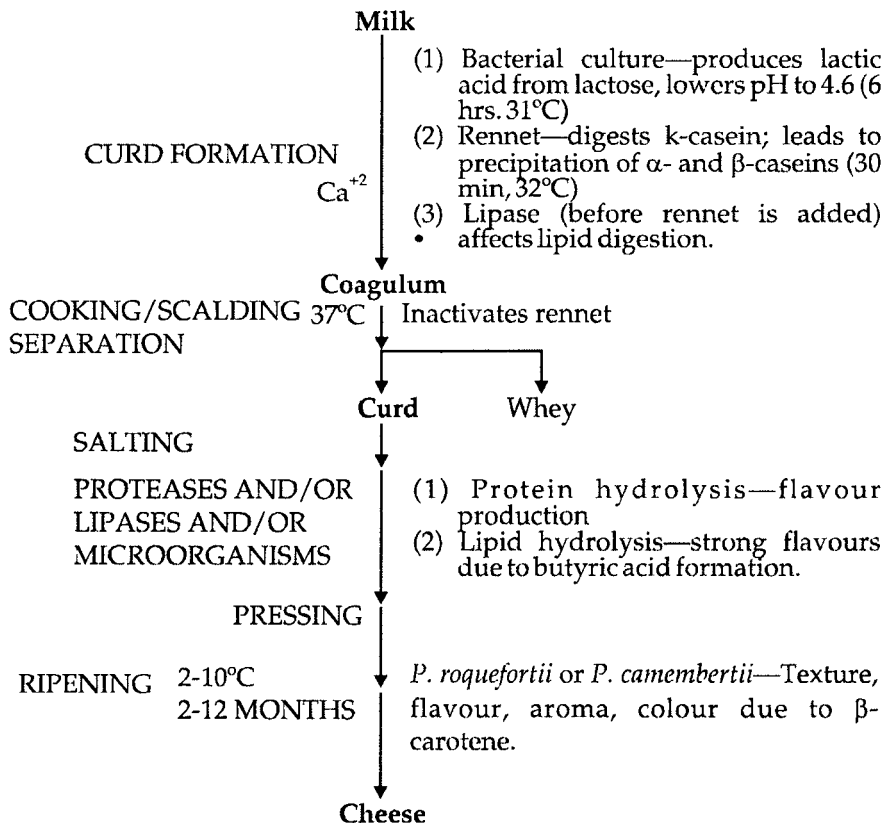
Fig. 12.2 : A schematic representation of the various steps in cheese production.

12.5 MICROBIOLOGICAL ASPECTS OF CHEESE-MAKING

12.5.1 Biological Agents

Following three biological agents are required for cheese making,

1. Milk
2. Starter Cultures
3. Rennet

Milk for cheese making: Although, cow's milk is the most commonly used source for cheese making all over the world, the use of milk obtained from buffaloes, goats, sheep, reindeer and mares is also practised in some parts of the countries like Italy, Greece, Egypt etc. Besides the composition of milk which varies with the species, breed and season, the quality of milk also has a direct effect on the quality and yield of cheese. The microbiological quality of raw milk reaching the cheese factory is controlled by the following major "H" factors: (i) health of the milch animal, (ii) hygiene during milk production (including hygiene of farm, personnel and equipment/utensils) and (iii) handling and refrigeration. Hygienic/ clean milk drawn from the udders of healthy animals carries only a few number of microorganisms (less than 10, 000 per ml). Maintenance of hygienic practices during milk production at farm is an important aspect of clean milk production. Cleaning and sanitization of milk contact surfaces determine the extent of contamination of milk after it has been drawn from the udders. The post-production hygienic handling of milk and proper refrigeration can check the proliferation of microorganisms in milk before cheese making. Milk should be held at around 4°C during transport (in insulated tankers) and in cheese plant (insulated silos).

(a) Microflora

(i) **Raw Milk**—In big dairies, milk is generally held in refrigerated conditions for 1-3 days before processing. The milk stored under these conditions may invariably contain predominantly psychrotrophic bacteria (over 1 million per ml). The common genera/ groups encountered are *Pseudomonas, Aeromonas, Alcaligenes,* lactic acid bacteria, Gram-positive sporeformers, coryneform bacteria, micrococci and coliforms. .The psychrotrophic bacteria may further increase in number due to their proliferation especially when the temperature of the refrigerated milk increases. The conditions facilitate the release of some heat resistant (surviving pasteurization) enzymes such as proteinases and lipases which may subsequently affect the quality as well as the yield of cheese.

(ii) **Pasteurized Milk**—The microflora of pasteurized milk used for cheese making comprises chiefly two types of bacterial flora apart from other microorganisms:

(a) Those surviving pasteurization (thermodurics) e.g. micrococci, enterococci, corynebacteria and sporeformers *(Bacillus* and *Clostridium).*

(b) Those entering after heat treatment (post-pasteurization contaminants) micrococci, enterococci, coliforms and lactic acid bacteria (lactococci, lactobacillis, pediococci and leuconostocs).

Besides bacteria, phages may also enter pasteurized milk if the environment of the cheese plant is contaminated. The heat resistant lipases and proteinases of the psychrotrophic bacteria mat also remain intact in pasteurized milk while the vegetative cells

may get killed. These heat stable enzymes in milk exert adverse effect on the overall quality and yield of cheese.

12.5.2 Starters for Cheese

The term *Starter* culture in context of cheese making refers to the selected microorganisms deliberately added to milk (or cream or a mixture of milk and cream) for initiating the desired fermentation that in turn controls the appearance, body, texture and flavour characteristics of cheese. In view of the vital role of starter cultures in cheese making, these are referred to as the 'Heart of Cheese'. The initiation of the cheese fermentation is due to acid production by lactic acid bacteria (cheese starters). Besides the primary function of acid production, these starters are also responsible for flavour production in cheese.

(A) **Functions of Starter Cultures**—In brief, following are the different primary and secondary functions of starter cultures (due to acid production) during cheese making.

Primary Functions:

(a) Acid production (due to lactic acid production).

(b) Flavour production due to production of flavour compounds as a result of breakdown of lactose, protein (proteolysis) and fat (lipolysis). Lactic acid also contributes to fresh acid flavour of curd cheeses.

(c) Eye formation (due to gas production as in certain varieties of cheeses like Swiss).

(d) Inhibition of undesirable (spoilage causing and pathogenic), microorganisms (due to production of acid and inhibitory substances of starter bacteria).

(e) Creation of suitable environment inside the cheese curd during maturation.

The secondary functions:

(i) Coagulation of milk by rennet.

(ii) Stimulating curd shrinkage and drainage of whey.

(iii) Suppression of growth of undesirable microorganisms during curd preparation and ripening.

(iv) Controlling the elasticity of the finished curd.

(v) Promotion of curd fusion into solid mass.

(vi) Controlling enzymatic changes during ripening which in turn affect the typical characteristics of cheese.

(B) **Classification of Cheese Starters**—Depending upon their acid producing abilities, starters (comprising bacteria and molds) may be lactic or non-lactic types.

(i) **Lactic Starters**—These are mainly lactococci, *Streptococcus thermophilus* and homo-fermentative lactobacilli.

(ii) **Non-lactic starters**—These include the following—

    (a) Propionibacteria in Swiss cheese.

    (b) Leuconostocs in Gouda and Dutch cheeses.

    (c) *Brevibacterium linens* in Brick type cheeses.

    (d) Molds in certain mold-ripened cheeses.

        — *Penicillium roqueforti* and *P. glaucuin* (blue-veined cheeses)

— *Penicillium camemberti* (Camembert cheese) Camembert

— *Geotrichum candidum*(Soft cheese, e.g. Brie and Camembert)

— *Mucor racemosus* (Norwegian skim milk cheese called Gammelost)

(C) Composition of cheese starters—Usually, a combination of starter microorganisms is used, the composition of which depends on the variety of cheese to be produced. The list of such starter combination used for different types of cheeses is given in Table 12.3.

Table 12.3 : Different starter combinations used for the preparation of different types of cheeses

Cheese	Starter composition
Cheddar	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* and *L. lactis* subsp. *diacetylactis*
Gouda	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *diacetylactis, Leuconostoc* spp.
Cottage	*L. lactis* subsp. *lactis, L. lactis subsp. cremoris, Leuconostoc* spp.
Swiss	*S. thermophilus, L. helveticus, Propionibacterium shermanii*
Brick	*L. lactis* subsp. *lactis, L. lactis* subs *p. cremoris, S. thermophilus, Brevibacterium linens*
Mozzarella	*S. thermophilus* or *S. faecalis* and *L. delbrueckii* subsp. *bulgaricus*
Blue (Roquefort)	*L. lactis* subsp. *lactis, Penicillium roqueforti*
Camembert	*L. lactis* subsp. *lactis, Penicillium camemberti*

(D) Criteria for selection of starter strains

(i) Temperature Sensitivity—The selection of mesophilic or thermophilic strains of bacteria for composing a cheese starter is determined by the temperature of cooking of curd, required during cheese-making, as has been shown in Table 12.4.

Table 12.4 : Starter selection based on cooking temperature of curd

Cooking temperature	Starter bacteria
Upto about 38°C	*L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris*
42°C to 46°C	S. *thermophilus* (active when curd is hot) + *L. lactis* subsp.
(e.g. Brick cheese)	*lactis* (activity starter as the curd cools to 38°C)
49° to 54°C	S. *thermophilus* + *Lactobacillus* spp. (e.g. Swiss cheese)
	(*L. delbrueckii* subsp. *bulgaricus, L. helveticus*)

(ii) Rate of Acid Production—Starter cultures used for cheese making may be slow, medium or fast acid producers. This can be tested by different starter activity tests like, Horall and Elliker New Zealand Activity test, or modified New Zealand Activity test.

(iii) Potential for Bitter Flavour—Bitterness in cheese has been directly associated with the starter strains that survive cooking temperature and continue to proliferate in the curd reaching high population. A simple test has been devised to classify bitter or non-bitter strains based on their growth in milk at normal cooking temperatures (37 to 38°C). The strains that remained viable at these temperatures could be regarded as potentially bitter

strains. However, a recent hypothesis suggests that almost all starter culture strains are capable of producing bitterness in response to selected manufacturing procedures.

(iv) Phage Sensitivity—The strain to be used as cheese starter is subjected to a formidable range of lytic phages which normally exist in the environment of cheese plant and hence only phage-insensitive strains or strains showing very low phage multiplication factors are selected.

12.5.3 Rennet

Rennet is added during cheese making to bring about coagulation of milk. This also helps in the ripening of cheese subsequently by causing proteolysis. Rennet is the term used to denote the enzyme preparation obtained by extracting the fourth stomach (called abomasum or veil) of suckling young ruminants (calves, lambs and goats). By far, the most extensively and traditionally used milk clotting enzyme for cheese-making is calf rennet (or more loosely referred to as animal rennet) which is obtained from 10-30 days old milk fed calves. Calf rennet was first commercialized in 1874 by Hansen (Denmark) and today it is available in liquid, powder and tablet forms. One part of rennet liquid (about 2% proteins) is used to clot about 5,000 parts of milk during cheese-making.

Rennet consists of mainly rennin and pepsin, principally the former bring responsible for milk clotting and the latter for proteolysis. Rennin (synonym: chymosin) a sulphur-containing protein, is labile to heat and alkali besides other physical and chemical agents and gets inactivated at 70°C in 14 minutes at pH 6.8-7.0. Its milk clotting activity is influenced by a number of factors, viz., concentration, temperature (optimum -41°C; acidity favours but alkalinity disfavours coagulation), calcium ions ($CaCl_2$ is added to hasten coagulation), inhibitory substances (e.g., albumin and globulin retard clotting);homogenization (promotes clotting of milk) and heat treatment (negative influence on milk clotting due to removal of calcium ions from casein).

(a) Mechanism of action—Rennet brings about clotting as well as proteolysis in milk during cheese-making.

(1) Clotting action—It occurs in two steps:

(i) Destabilizing step—Casein micelle is destabilized by disturbance of its colloidal nature as a result of action of rennet on, k-casein fraction of casein as indicated below:

$$\text{Casein micelle} \xrightarrow{\text{Rrennet}} \text{para k-casein + glycomaropeptide}$$
$$\text{(k-casein)}$$
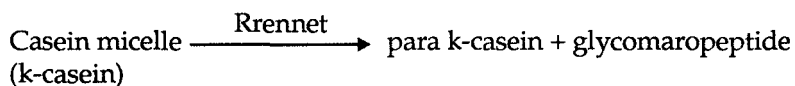
The above reaction is due to cleavage of peptide linkage between amino acids at 105 and 106 positions in the peptide chain of k-casein, which has a total length of 169 amino acids.

<div align="center">

k-casein

Pyroglutamyl ⟶ Phe ⟶ Met ⟶ Valine

1   105   106   169

para k-casein   Rennet   k-caseinomacropeptide

</div>

(ii) **Coagulation step—**

$$\text{para k casein} + \text{Ca} \longrightarrow \text{calcium para k-casein}$$
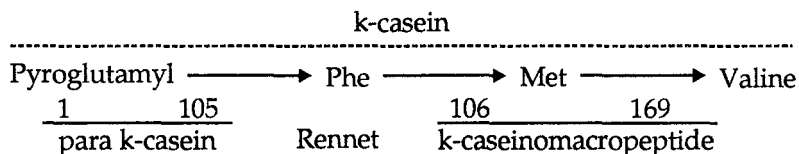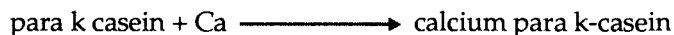
(2) **Proteolytic action—**Milk proteins are broken down to peptides of varying lengths due to rennet action mainly during ripening of cheese;

$$\text{Protein} \xrightarrow{\text{Rennet}} \text{Peptides}$$
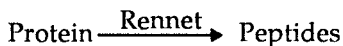
(b) **Rennet Substitutes—**The increased demand and production of cheese the world over coupled with the decreased culling and slaughtering of calves due to improved management practices has resulted in a substantial shortage of calf rennet. The resulting high cost combined with certain sentimental and religious reservations of vegetarian population in consuming the cheese made with animal rennet in some countries like India and Israel have stimulated interest in rennet substitutes from non-animal sources like plants and microorganisms. Among the microbial milk clotting enzymes investigated so far, a few have been introduced in the market commercially by some foreign firms. However, the microbial rennets have the following limitations:

(i) Low milk clotting to proteolytic activity ratio

(ii) High retention of the enzyme in the curd

These properties tend to cause hydrolysis of caseins to the point of forming bitter peptides during cheese ripening. Although, researches have been carried out to correct these defects in microbial rennet substitutes, the calf rennet is still the most accepted milk coagulant for cheese making. Recently, recombinant rennet has been produced by recombinant technology by cloning calf rennet genes into suitable microbial hosts and the preparation is likely to be commercially successful.

12.6 PUBLIC HEALTH CONCERNS/DISEASES THROUGH CHEESE

The microenvironment is unfavourable for the growth of pathogens in cheese due to (1) low initial pH, (ii) relatively high salt-in-water phase, (iii) low moisture content, (iv) low temperature of storage, and (v) presence of inhibitory starter metabolites. However, if proper care is not taken, a variety of pathogens can be transmitted through consumption of cheese. They come either from animal via improperly pasteurized milk, human handlers, rodents, and environment.

Following ill-healths are possible through cheese—

(1) Food poisoning caused by *Staph. aureus* account for the majority of outbreaks attributed to cheese. Staphylococcal enterotoxin is resistant to heat and other conditions. Hence even if the pathogen die by heat treatment, the toxins persist and cause food poisoning.

(2) Typhoid fever due to *Salmonella* infection through contamination in cheese.

(3) Other animal-derived pathogens like *Brucella abortus* and *Myco. tuberculosis* are problematic when raw milk is used for cheese making.

(4) *Listeria monocytogenes* and *Yersinia enterocolitica* are able to grow at low temperature (3-5°C) and cause fatal illnesses.

(5) *E. coli* may grow in soft cheeses where moisture and pH conditions are favourable.

(6) Toxic metabolites of molds, called mycotoxins have been detected in cheese. Especially, there is greater risk of aflatoxin produced by *Aspergillus* and patulin produced by *Penicillium* if mold growth on cheese is not properly controlled.

The risk of all pathogens can be minimised by following control measures—

(1)   Strict hygienic precaution to minimise entry of pathogens in cheese.

(2)   Proper pasteurization of milk.

(3)   Ensuring rapid starter growth during cheese making.

(4)   Ripening for at least 60 days, as all pathogens die slowly during ripening process.

12.7 MICROBIOLOGICAL SPOILAGES OF CHEESE AND THEIR CONTROL

Cheese is extremely susceptible to microbiological spoilage. However, there are a number of limiting factors inherent in cheese which can influence the microbiological quality of cheese. These factors include moisture content, residual lactose content, oxygen level, salt concentration and pH of the product. The following defects in cheese can be attributed to microbial activity.

12.7.1 Mold Growth

Except in certain mold ripened cheeses, mold growth in a cheese variety is considered undesirable because it spoils the appearance of the product and also produces musty off-flavour besides the possibility of mycotoxin production. The molds commonly encountered in cheese ripening rooms and involved in spoilage include *Alternaria, Aspergillus, Cladosporium, Monilia, Mucor* and *Penicillium* as well as *Geotrichum*. The growth of yeasts and molds in cheese rooms can be controlled by practising proper cleanliness and hygiene and by controlling the temperature and humidity. Yeast and mold growth on cheese surfaces can be controlled by treating the packaging materials with fungistatic agents or fungicides like organic acids, antibiotics and other antifungal agents.

Installation of UV-lights can also restrict mold growth. Vacuum packaging of cheese blocks, which is currently followed by many cheese producers, is a very effective control measure against molds.

Certain non-toxic antibiotics like pimaricin or nisin can be incorporated in cheese to prevent of spoilage of cheese.

Flavour defects—Uncontrolled microbial growth can produce several undesirable flavours in cheese. Bitterness is very common defect produced by proteolytic bacteria. Cheesy, putrid, fruity and rancid odours are also related to improper protein and fat decomposition by microorganisms. Yeasty flavour can be implanted by excessive growth of yeasts.

12.7.2 Gas Formation

The gas producing organisms such as coliform and Clostridia already present in milk or entering as contaminants in the curd may cause undesirable gas formation during manufacture or ripening of cheese. The nature of gas defect depends on the number and types of these organisms which may result into defects like curd floating, early blowing, late blowing and excessive openness. The gas formation in cheese can be controlled by using a good quality milk and following proper conditions of heat treatment. Other preventive measures include keeping milk free from contamination with these organisms, attaining faster acid formation in curd, adequate salting of cheese and by using nisin producing *L. lactis* subsp. *lactis*.

12.7.3 Rind Rot

This defect in cheese is the result of moisture accumulation on cheese surface which can promote the growth of surface flora-like film yeasts, molds, proteolytic bacteria and others. Such microbial growth causes softening and discoloration of cheese surface accompanied by off-flavours. Rind rot formation can be controlled by keeping the cheese surface dry and by turning the cheese at regular intervals.

12.7.4 Discolorations/Colour Defects

Colour defects appear on surface or in the interior of cheese. This defect is caused by the growth of molds like *Aspergillus niger* which produces black spots on hard cheese; *Sporendonema casei* which causes red spots on blue cheese. The occurrence of coloured spots inside the cheese mass is rare though possible in some cheeses due to bacterial growth e.g. rusty spots in cheddar is caused by pigmented variants of *Lactobacillus plantarum* and *L. brevis* and colour spots in Swiss cheese due to pigmented species of *Propionibacterium*. The control measures include maintenance of good sanitary and hygienic conditions in the cheese room and by controlling the temperature and humidity.

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13

COMMERCIAL MANUFACTURE OF CHEESES

13.1 INTRODUCTION

Cheese is a food manufactured from milk. For many cheese makers, there is an art to making cheese. To cheese manufactures, it is commonly a routine, strictly controlled process, and has been refined through strict adherence to manufacturing guidelines and careful selection of specific lactic bacteria and ripening microorganisms.

Imagine, a long time ago, when humans first tasted that odorous morsel covered with colourful molds, yeasts and bacteria. But now consider a world without Roquefort, Stilton, Lumburger, or Mozzarella. Boring! Unthinkable!

Its early history of development is not known but according to ancient records, cheese has been used as a food for over 4000 years. It was made during Biblical times and it is believed that the knowledge of cheese making was brought originally from Asia to Europe and was introduced into many parts of Europe when the Roman Empire flourished. During the Middle Ages, important contributions to cheese manufacture were made by monks in the monasteries and mention of cheese is made in the monastery records.

Until about the middle of the 19th century, cheese was a farm industry wherein cheese was made from the surplus milk produced on the farm. The first cheese factory in the U.S.A. was built by Jesse Williams in New York in 1851.

Manufacture of some popular cheeses are schematically explained in this chapter.

13.2 DEFINITIONS OF COMMERCIAL VARIETIES OF CHEESE

The U.S. Department of Agriculture has adopted the following definitions and standards for cheese.

WHOLE MILK CHEESE

Cheddar Cheese, American Cheese, American Cheddar Cheese—The cheese made, by the Cheddar process from heated and pressed curd obtained by the action of rennet

on whole milk. It contains not more than 39 per cent of water, and, in the water-free substance, not less than 50 per cent of milk fat.

Pineapple Cheese—The cheese made by the pineapple Cheddar cheese process from pressed curd obtained by the action of rennet on whole milk. The curd is formed into a shape resembling a pineapple, with characteristic surface corrugations, and during the ripening period the cheese is thoroughly coated and rubbed with a suitable oil, with or without shellac. The finished cheese contains, in the water-free substance, not less than 50 per cent of milk fat.

Brick Cheese—The quick-ripened cheese made by the brick-cheese process from pressed curd obtained by the action of rennet on whole milk. It contains, in the water-free substance, not less than 50 per cent of milk fat.

Stilton Cheese—The cheese made by the Stilton process from unpressed curd obtained by the action of rennet on whole milk, with or without added cream. During the ripening process a special blue-green mold develops, and the cheese thus acquires a marbled or mottled appearance in section.

Gouda Cheese—The cheese made by the Gouda process from heated and pressed curd obtained by the action of rennet on whole milk. The rind is coloured with saffron. The finished cheese contains, in the water-free substance, not lees than 45 per cent of milk fat.

Neufchatel Cheese—The cheese made by the Neufchatel process from unheated curd obtained by the combined action of lactic fermentation and rennet on whole milk. The curd, drained by gravity and light pressure, is kneaded or worked into a butter-like consistence and pressed into forms for immediate consumption or for ripening. The finished cheese contains, in the water-free substance not less than 50 per cent of milk fat.

Cream Cheese—The unripened cheese made by the Neufchatel process from whole milk enriched with cream. It contains, in the water-free substance, not less than 65 per cent of milk fat.

Roquefort Cheese—The cheese made by the Roquefort process from unheated, unpressed curd obtained by the action of rennet on the whole milk of sheep, with or without the addition of a small proportion of the milk of goats. The curd is inoculated with a special mold *(Penicillium roqueforti)* and ripen with the growth of the mold. The fully ripened cheese is friable and has a mottled or marbled appearance in section.

Gorgonzola Cheese—The cheese made by the Gorgonzola process from curd obtained by the action of rennet on whole milk. The cheese ripens in a cool, moist atmosphere with the development of a blue-green mold and thus acquire a mottled or marbled appearance in section.

WHOLE MILK OR SKIM MILK CHEESE

Edam Cheese—The cheese made by the Edam process from heated and pressed curd obtained by the action of rennet on whole milk or on partly skimmed milk. It is commonly made in spherical form and coated with a suitable oil and a harmless red colouring matter.

Swiss Cheese—The cheese made by the Emmenthaler process from heated and pressed curd obtained by the action of rennet on whole milk or on partly skimmed milk. It is ripened by special gas-producing bacteria, causing characteristic "eyes" or holes. The finished cheese contains, in the water-free substance, not less than 45 per cent of milk fat.

Camembert Cheese—The cheese made by the Camembert process from unheated, unpressed curd obtained by the action of rennet on whole milk or on slightly skimmed milk. It is ripened by the growth of a special mold *(Penicillium camemberti)* on the outer surface. The finished cheese contains, in the water-free substance, not lees than 45 per cent of milk fat.

Brie Cheese—The cheese made by the Brie process from unheated, unpresaed curd obtained by the action of rennet on whole milk, on milk with added cream, or on slightly skimmed milk. It is ripened by the growth of a special mold on the outer surface.

Parmesan Cheese—The cheese made by the Parmesan process from heated and hard-pressed curd obtained by the action of rennet on partly skimmed milk. The cheese, during the long ripening process, is coated with a suitable oil.

Cottage Cheese—The unripened cheese made from heated or unheated, separated curd obtained by the action of lactic fermentation or rennet, or a combination of the two, on skimmed milk, with or without the addition of buttermilk. The drained curd may be enriched with cream, and salted or otherwise seasoned.

PASTEURIZED CHEESE

Pasteurized Cheese, Pasteurized-blended Cheese.—The pasteurized product made by comminuting and mixing, with the aid of heat and water, one or more lots of cheese into a homogeneous, plastic mass. The unqualified name "pasteurised cheese," "pasteurized-blended cheese," is understood to mean pasteurized Cheddar cheese, pasteurized-blended Cheddar cheese, and applies to a product which conforms to the standard for Cheddar cheese. Pasteurized cheese, pasteurized-blended cheese, bearing a varietal name is made from cheese of the variety indicated by the name and conforms to the limits for fat and moisture for cheese of that variety.

PROCESS CHEESE

Process Cheese—The modified cheese made by comminuting and mixing one or more lots of cheese into a homogeneous, plastic mass, with the aid of heat, with or without the addition of water, and with the incorporation of not more than 3 per cent of a suitable emulsifying agent. The name "process cheese" unqualified is understood to mean process Cheddar cheese, and applies to a product which contains not more than 40 per cent of water and, in the water-free substance, not lees than 50 per cent of milk fat. Process cheese qualified by a varietal name is made from cheese of the variety indicated by the name, and conforms to the limits for fat and moisture for cheese of that variety.

13.3 BASIC STEPS IN COMMERCIAL CHEESE MAKING

The process of cheese making involves two basic phenomenon: (i) Preparation of cheese curd and (ii) ripening of cheese which are accomplished through a series of steps, namely, preparation of milk, addition of starter, rennet coagulation of milk, processing of the curd (cutting, healing, foreworking, predrawing, cooking, agitation, washing, draining, matting, cheddaring, milling, mixing, hooping, pressing), salting of cheese curd and ripening/curing of cheese curd into final product.

13.3.1 Preparation of Milk

Milk to be converted into cheese, is subjected to different treatments like clarification/ filtration, bactofugation, H_2O_2 treatment, standardization, homogenization, heat treatment etc.

13.3.2 Preparation of Milk Coagulum

Coagulation of milk is the transformation of milk to a solid or semi-solid (gel) state. It is brought about by acid or rennet or a combination of both, depending upon the variety of cheese to be prepared. Acid coagulation is practised only in certain types of cottage cheese. The general practice is to use lactic acid bacteria (for acid production) followed by rennet addition because the increase in acidity promotes coagulation of milk by rennet.

(a) Addition of starter and ripening (souring)—Pre-selected starter culture in a desired combination is added to cheese milk, usually before the whole amount of milk has been transferred into the vat, either by adding directly to the vat or by injecting into the milk-line with a pump. A fresh starter culture is normally added (at the rate of 0.5 to 1.0%) although frozen concentration or freeze dried (lyophilized) powder may also be used. The inoculated milk is then allowed to ripen (sour) at 30-31°C for 30 to 60 mm. The ripening process permits starter bacteria to acclimatize to the new environment (vat milk) which leads to production of lactic acid sufficient to activate rennet besides other secondary functions. At normal pH of milk (6.6), most of the calcium is in colloidal form but as the acid is produced due to starter activity, this calcium shifts to the soluble (ionic) form and therefore, becomes functional during the secondary stage of coagulation process. The other secondary functions of lactic acid production in cheese include facilitation of whey expulsion, suppression of undesirable microorganisms and control of texture and ripening changes.

(b) Renneting and Coagulation—Rennet is diluted (usually 20 to 40 times) with fresh potable chlorine free water before addition to ripened milk for easy and uniform distribution. The rate of rennet addition depends on the variety of cheese to be prepared. Rennet solution is added with thorough stirring followed by the undisturbed holding (usually for about 30 min.) of milk for obtaining homogeneous compact curd. The length of time for coagulation (set time) and firmness of the coagulum are controlled by composition of milk, acidity of milk (availability of calcium), temperature of milk and amount of rennet added.

13.3.3 Transformation of Coagulum into Cheese Curd (whey expulsion steps)

The rennet coagulum of milk is transformed into fresh cheese curd by whey removal, using a suitable combination and sequence of the following steps, viz., cutting, healing, foreworking, predrawing, cooking and stirring, draining and washing, cheedaring, milling, mixing, hooping and pressing. Whey removal is facilitated by (i) acid and (ii) heat, besides the mechanical processing, causing the shrinkage (syneresis) of the curd which determines the body texture of the final product.

13.3.4 Starter Proliferation and Activity

From microbiological point of view, the activity of starter bacteria continues throughout the moisture expulsion steps of cheese-making. The starter bacteria which had multiplied during ripening of milk get trapped in the rennet coagulum. On cutting the coagulum, the majority of the starter population is retained in the curd where two effects are noticeable, viz., the proliferation of starter bacteria and lactic acid production from lactose. The rate of these activities further increases during the cooking stage due to elevation of temperature nearing their optimum growth temperature. As the starters multiply, lactose is depleted resulting into production of lactic acid in the curd. Besides depletion of lactose,

the acid produced in the curd causes chemical changes in casein and solubilizes the colloidal calcium phosphate. Due to further transformation of these constituents, whey constituents including lactose simultaneously diffuse back into the curd which further leads to microbial proliferation and acid production. Hence, longer the retention of curd in the whey, lower will be the pH of the curd due to continuous availability of lactose in the curd. Although the total acid production is more in the curd than in whey, the pH of the former does not decline as rapidly as that of the latter due to concentration of casein in the curd. The rate of starter proliferation varies with the type of cheese e.g., in Cheddar cheese it is maximum during curd-making stage.

The lactic acid produced by starter activity has twofold advantage: (a) helps in moisture expulsion and (b) effects the chemical and physical composition of the curd.

13.3.5 Salting of Cheese Curd

The different varieties of cheese are salted at the rate of 1 to 10% of sodium chloride. Salt is added to cheese either as dry salt or as brine by one of the following methods or their combination. Dry salt may be added to cheese milk, dusted on the milled curd in the vat just before hooping (e.g. in cheddar cheese), rubbed on cheese surface (e. g. in Brie and Camembert varieties) or added to creaming mixture (e.g. in creamed cottage cheese). Brine may be sprayed on the curd surface before hooping or cheese curd may be floated in saturated (90-100%) salt brine tanks (e.g. in Mozzarella, Swiss and Romano) after hooping. In any of the above applications, salt diffuses in the whole cheese mass and gets uniformly distributed. The distribution is affected by different factors, namely, moisture content of the cheese, concentrations of salt/brine applied, time and temperature of exposure and surface area to volume ratio of the cheese.

13.3.6 Ripening of Cheese

Ripening (also referred to as curing, maturing or ageing) of cheese is a process of storage of fresh cheese curd at suitable temperature(s) till it transforms into a finished product of desirable body, texture and flavour. The transformation involves the activity of ripening agents, viz., microorganisms on various cheese constituents (mainly lactose, protein and fat) to cause physical, chemical (biochemical) and microbiological changes in the product.

(i) Ripening agents—Cohn was the first, to correlate the cheese maturation with bacterial activity in 1875. Most of the microorganisms isolated by him from cheese were rods which when inoculated in milk produced cheesy aroma. Subsequently, a number of theories were proposed on cheese ripening. It is believed that cheese maturation is an enzymatic process wherein enzymes are liberated on autolysis of microorganisms. Microorganisms and enzymes are, therefore, believed to constitute the two basically required biological agents for cheese ripening.

(A) Microorganisms—The microorganisms participating in the ripening event can be placed in the following two groups: (a) starter, and (b) non-starter microorganisms.

(a) Starter microorganisms—This category includes the microorganisms intentionally added to cheese milk or introduced in or on the pressed cheese curd to produce the characteristic product of uniform quality after ripening. Starter flora could be further grouped into lactic and non-lactic types, depending on their abilities for acid production.

(b) **Non-starter microorganisms (adventitious microflora)**—This group refers to microorganisms which are not added deliberately by the manufacturer but gain access to the cheese vat as contaminants during different stages of cheese-making. These microorganisms are believed to come from the milk and milk contact surfaces, worker's hands and clothes and equipments. Indication of a definite role of non-starter flora in flavour development trigger a series of investigations with individual groups of adventitious microflora, referred to as 'reference flora' during aseptic cheese trials. Adventitious microflora only aid in faster and more intense flavour development because of the fact that in the absence of non-starter flora, a longer time was required for the development of full *'Cheddar'* flavour.

(B) Enzymes—Various enzymes (carbohydrases, proteinases, lipases etc.) responsible for bringing about the ripening changes in cheese have been believed to originate from milk, rennet and microorganisms sources.

(ii) Ripening changes—The changes due to enzymatic activity during ripening are summarized below:

13.3.7 Physical Changes

(a) **Body**—The body of cheese, which refers to its consistency, covers the following characteristics: firmness, elasticity, plasticity and cohesiveness. The fresh cheese curd with initial tough and rubbery consistency gets transformed into a product with softer or crumbly (if moisture content is low) body, a change caused by enzymatic solubilization of casein portion of cheese. On the other hand, an initial crumbly and brittle curd, which is obtained when there is high acid development during cheese-making results in cheese with "short" body, i.e. the product lacks elasticity as it breaks on stretching.

(b) **Texture**—In cheese, texture refers to the extent of openness due to presence or absence of 'holes' or spaces in the cheese mass. Hence, there may be a 'close texture' (when no holes are present) or an 'open texture' (when holes are present). In many cheese varieties (e.g. Cheddar) an almost close texture is desirable and a more than slight openness is considered as a defect. In other cheeses (e.g. blue-veined varieties), open texture is desired for growth and proliferation of molds throughout the product. Openness due to moderate gas production is acceptable in some (e.g. Brick, Gouda, Limburger) and essential in others (e.g. Emmental, Gruyere) varieties of cheese.

(c) **Flavour**—Production of desirable flavour in cheese is the primary change during ripening process. Although, the background of flavour is provided by the presence of lactic acid and salt in cheese curd, the typical flavour is developed due to release of certain flavour compounds on microbial and enzymatic breakdown of protein, fat, lactose and other constituents like lactic acid/lactates and citrate. Hence, it is the complex mixture of various flavour components (volatile fatty acids and their esters or ketones, amino acids and other protein derivatives, carbonyls etc.) in balanced proportions that constitutes the characteristic flavour of different varieties of cheese.

13.3.8 Chemical/Biochemical Changes

Enzymes derived from microorganisms, rennet and milk act mainly on lactose, protein and fat to produce a variety of biochemical/chemical changes in cheese during

ripening. These changes may be of two types: primary and secondary. In primary changes, breakdown of the three main constituents of cheese (Lactose, protein and fat) leads to the accumulation of lactic acid, amino acids and fatty acids (primary compounds). In case of secondary changes, enzymes primarily from microorganisms act on the primary compounds to generate simpler compounds. The secondary reactions from lactate and pyruvate yield propionic acid (in Swiss cheese), citric acid, acetic acid, acetyl methyl carbinol, diacetyl, acetaldehyde, ethanol and other compounds including TCA cycle intermediates. From amino acids are derived amines, keto acids, aldehydes, cystine-cysteine-cysteic acid-taurine, serine-pyruvic acid, methionine, trimethyl keto butyric acid, glutamic acid-glutamine, aspartic acid-asparagine, tyrosine-tyramine, glycine-acetic acid-acetyl CoA etc. From fatty acids like butyric, caproic, caprylic and capric are derived acetoacetic acid-acetone, β-keto acids-β keto caproic acid-methyl ketones, esters etc. In the degradation process, certain gases are released, viz., NH_3, H_2S and CO_2. Ammonia and H_2S contribute directly to cheese flavour whereas CO_2 contributes indirectly.

13.3.9 Microbiological Changes

Microbial changes during cheese ripening depend on the mode of ripening which may differ with the type of cheese. Basically, there are two modes of ripening used either alone or in combination.

    (i)   In hard and some semi-hard varieties of cheeses, ripening conditions are such that microbial/enzymatic activity is limited inside the cheese mass and surface growth of microorganisms is discouraged. Irrespective of the size and shape of the cheese block, ripening proceeds evenly and uniformly throughout the product due to microbial activity.

    (ii)   In all soft and some semi-soft cheeses, ripening conditions are such that surface growth of microorganisms is encouraged e.g. growth of *Brevibacterium linenes* in bacterial surface ripened cheeses and of molds (e.g. *Penicillium camemberti*) in mold surface ripened cheeses. The surface microorganisms release enzymes that diffuse into the cheese mass. This mechanism of ripening changes progressively from the surface towards the centre necessitates small size and flat shape of the cheese blocks in these varieties.

    (iii)   In other mold ripened (internal) cheeses, viz., blue veined cheeses, a combination of the above two modes of ripening is used. In the early stages of ripening, microorganisms and their enzymes bring about changes inside the cheese mass. Air is then admitted into the block by making holes. Molds (e.g. *Penicillium roqueforti*) either inoculated or coming naturally from the atmosphere of the ripening room then act after proliferation in the cheese and produce various characteristic changes in flavour and appearance due to biochemical activities (lipolytic and proteolytic).

13.3.10 Accelerated Cheese Ripening

The conventional method of ripening of cheeses particularly hard varieties is time and space consuming, cumbersome and, energy intensive. The prolonged curing is thus uneconomical as it not only locks up the money invested but also increases the cost of production due to the application of refrigeration for maintaining the required temperature.

Recently, successful attempts have been made to develop accelerated processes of cheese ripening for obtaining the original natural characteristic flavour by adopting the following two approaches:

(i) **Biological**—Addition of ripening agents (enzyme and/or microorganisms), cheese slurries and flavouring compounds or biochemicals.

(ii) **Technological**—Manipulation of manufacturing techniques (e.g. adopting strategies suiting higher retention of moisture in curd) and curing conditions (e.g. temperature, humidity).

The following description will mainly highlight the use of biochemical and microbiological approaches for achieving accelerated cheese ripening.

1. Addition of enzymes—The potentially useful enzymes for cheese ripening such as proteases, lipases, lactases (β-galactosidase) and decarboxylases could be added exogenously to milk or curd for hastening up the enzymatic changes in cheese during ripening. Usually, proteases (proteinases and/or peptidases) and lipases are used for the purpose as breakdown of proteins and fat mainly provides the pool of flavour precursors during ripening. Generally, addition of lipase is desirable in blue-veined varieties as these are strongly flavoured cheese varieties. In cheddar type varieties, addition of lipases tends to produce off-flavours mainly rancidity and proteinases, if used alone, produce bitterness. However, a combination of proteinases and peptidases causes accelerated flavour production without much bitterness. Recently, β-galactosidases have been claimed to accelerate production of cheddar flavour. When the commercial lactase preparation was added to cheese milk, the proteolysis increased which could either be due to stimulation of starter multiplication (as lactase converts lactose to simple sugars) resulting in higher proteinase content or due to presence of contaminating proteinases in the lactase preparation.

Some new commercial preparations of the degradative enzymes, namely, Naturage (protease + lipase), Flavourage (lipase + protease), Maxilact (β-galactosidase) etc. are available for accelerating cheese ripening. Cheese made from buffalo milk is very slow in its flavour and body and texture development during ripening. However, this problem could be overcome by the judicious use of microbial rennet substitutes instead of calf rennet as the former cause faster degradative changes during ripening.

2. Addition of desirable microorganisms—Nowadays with a view to increase the concentrations of desirable enzymes in cheese, several efforts to either increase the population of starter bacteria or to incorporate the non-starter microorganisms with high proteolytic activities have been made for hastening the ripening process.

Lactococci have slow and fast variants according to the rate of acid production and proteolysis. A mixture of 2:1 of slow and fast strains of these bacteria has been recommended for producing a good flavoured cheese. Although, the Lactobacilli virtually take no part in acid development stage of cheese making, these may hasten proteolysis during ripening stage and thus, accelerate the process to produce high quality cheese.

Mutants of lactic acid bacteria, viz., lac- (lacking the ability to convert lactose to lactic acid) such as *Lactococcus lactis* subsp. *lactis* and X-ray mutants of lactobacilli with enhanced proteolytic activity have been tried for accelerated cheese ripening with varying success. However, more work is required on such mutants to establish their effect. The latest attempts in using genetically modified starters are towards development of recombinants with improved and stable degradative abilities through genetic engineering. This area of

'Biotechnology' holds a great promise in improving and accelerating the cheese ripening process as a whole.

3. Incorporation of flavouring components—Successful attempts have been made for inducing quick flavour development in cheese and cheese spreads by adding a preselected mixture of flavouring compounds. On blending cheese curd with a mixture of methyl ketones and fatty acids, a distinct cheddar cheese flavour could be obtained just in 3 weeks time. Similarly, a cheesy flavoured product could be obtained by mixing a food with synthetic alkylamines such as propylamine, butylamine, 2-methyl propylamine, pentylamine, 2-methyl butylamine, 3-methyl butylamine and hypochloride salts in a final concentration of 0.1 to 80 mg per kg. It has also been claimed that a mixture of 3 amino acids out of glutamic acid, alanine, proline and methionine considerably enhanced cheese flavour when their concentration in the final product was twice than that of other free amino acids.

13.4 FRESH CHEESES—QUARK

They are natural, unripened products. They are manufactured from pasteurized or tempered milk of adjusted fat content by the use of souring cultures and rennet or rennet substitutes or with souring cultures alone by the precipitation of the casein and the separation of whey, and are marketed in the fresh state. Keepability can be improved by heat treatment (Puhan, 1979; Kielwein and Melling, 1978).

Fresh cheese preparations are obtained by incorporating flavouring additives in the cheese mass. The fresh cheeses include quark and granular fresh cheese (cottage cheese).

Quark (fresh cheese, fresh cream cheese, white cheese, fresh double-cream cheese, layer cheese).

Quark forms the main fresh-cheese product in large parts of Europe. It is manufactured only from pasteurized skim milk the fat content of which has been adjusted or, for the higher-fat grades, from milk with the later incorporation of cream. Quark possesses a white to cream-yellow uniformly soft, smooth to paste-like dough without any skin or rind whatever, without the release of whey, and without granular or gritty structures and a clean slightly lactic acid taste (Loos and Nebe, 1980) Quark is prepared with souring cultures and small amounts of rennet (sour-rennet quark) or souring cultures alone (sour-milk quark). In the low-temperature souring process at 20 to 30°C (time of souring 15 to 22 hours), mesophilic cultures, creamery cultures, or single-strain cultures of *S. cremoris* are used. In the warm souring process àt 38 to 40°C (souring time 3 to 5 hours), thermophilic lactic acid bacteria *(S. thermophilus* and *L. bulgaricus)* are used.

Manufacture of quark (Fig. 13.1)

Preparation of the milk—Milk of adjusted fat content from the primary stock tank is heated to approximately the temperature of pasteurization, homogenized, and then pasteurized in the pasteurizer at 72°C for 15 seconds or at 85 to 95°C for a few seconds, cooled in the cooler to 20 to 27°C or 38 to 42°C, according to the process being used, and pumped into ripening tanks with a capacity of 15000 to 50000 L.

Souring and curdling—In the souring tank, 0.5 to 2% of the souring culture in the low-temperature process and 3 to 4% in the warm souring process is added, with, if required, the rennet preparation, and the mixture is thoroughly stirred for about 2 minutes. Then the milk is left to rest until it has curdled. Small amounts of rennet are said to accelerate the coagulation through slight proteolysis and therefore also to have a favourable effect on the

stability of the consistency of the casein gel. In no case may the rennet in the quark bring about a far-reaching decomposition of the casein through proteolytic cleavages going beyond this.
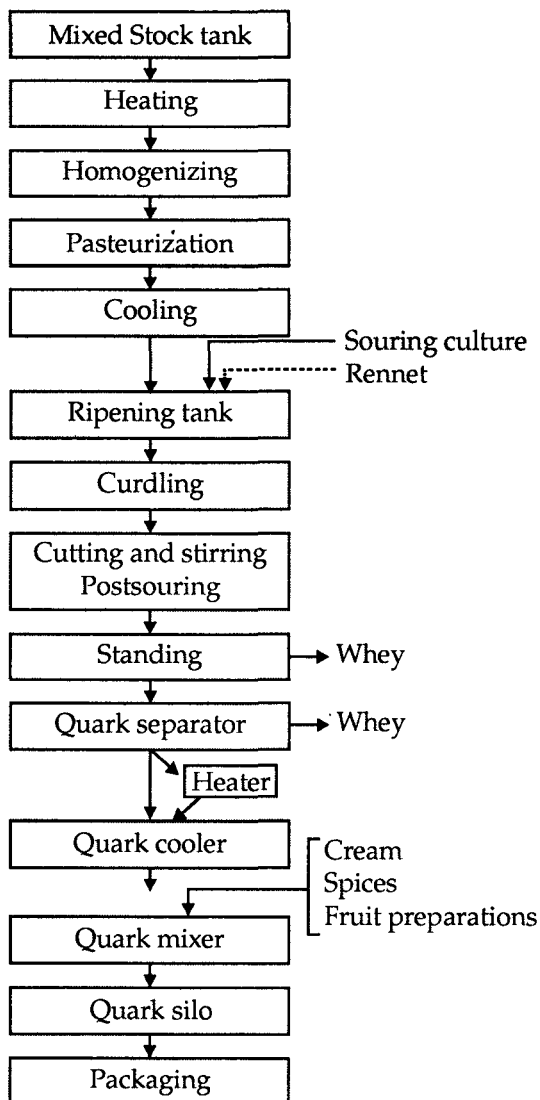
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│  Mixed Stock tank   │
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┌─────────────────────┐
│      Heating        │
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┌─────────────────────┐
│    Homogenizing     │
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          ↓         ──── Souring culture
                    ┄┄┄┄┄ Rennet
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│   Ripening tank     │
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│     Curdling        │
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┌─────────────────────┐
│ Cutting and stirring│
│     Postsouring     │
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┌─────────────────────┐
│     Standing        │──→ Whey
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│   Quark separator   │──→ Whey
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             │ Heater │
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│    Quark cooler     │    ┌ Cream
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│    Quark mixer      │
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│    Quark silo       │
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│     Packaging       │
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Fig. 13.1 : Manufacture of edible quark.

Cutting and stirring—After 8 to 12 or 2 to 3 hours, When a pH of 4.8 has been reached, the coagulum is cut by an integrated cutting and stirring device in the souring tank into sections with an edge length of about 10 to 20 cm and is slowly stirred. In another 6 to 8 or 1 to 2 hours, with souring proceeding further, whey slowly separates out and the coagulum sinks in the whey.

Separation—As soon as the pH has fallen to 4.65 to 4.6, the stirring is stopped and the supernatant whey is run off. After this, the quark and the residual whey are removed

from the tank. The quark can now be heat-treated at 60°C to stop the activity of the rennet and to kill re-contaminants and souring bacteria, i.e., to prolong keepability. Then, at a temperature of 30⁰C, the substrate passes through the quark separator for the elimination of more whey and for the adjustment of the desired dry-matter content of the product. After this the quark is cooled to 4 to 8°C.

Mixing—The quark is processed to a uniform consistency in a kneading and mixing machine. Pasteurized cream, spices, fruit preparations may be incorporated at the same time. The finished product is first stored in a quark silo and is then fed into packaging devices where filling is carried out with the greatest possible efforts to avoid recontamination. Quark pumps, piping, separators, and mixing devices of the unit must be so designed that the acid-coagulated casein micelles are not damaged by shearing forces, which would lead to a continuing release of whey (Dolle, 1977; Spreer, 1978; Kessler, 1976).

13.5 COTTAGE CHEESE (GRANULAR FRESH CHEESE)

Cottage cheese is a fresh-cheese speciality. In the USA, it forms the main fresh cheese product. Edible quark is largely unknown there.

Cottage cheese is obtained from pasteurized skim milk with the addition of souring cultures, and also with the addition of rennet, by the acid coagulation of casein, and by special further treatment as a granular product and to improve its taste it is mixed with cream and with the addition of common salt, and filled into containers.

Manufacture of cottage cheese (Fig. 13.2)

Preparation of the milk—Selected skim milk is homogenized and subjected to gentle pasteurization (72 to 75°C for 15 seconds).

Souring—The souring cultures used are mesophilic mixed cultures and also creamery cultures. These populations consist overwhelmingly of organisms of S. *cremoris*, a small proportion of S. *lactis* and from 1% to a maximum of 3% of aroma-forming agents (S. *lactis* subsp. *diacetylactis* and/or *Leuconostoc cremoris*). Larger proportions of aroma-forming agents in the culture adversely affect the product by the pronounced formation of gas. These cultures are added to the milk. To facilitate curdling and the release of whey and to improve the granular texture by proteolysis, small amounts of rennet may be added to the milk.

The amount of starter culture and the temperature determine the time until the milk curdles.

Milk, starter culture, and any rennet are mixed directly in the cheese vat and the mixture is left unstirred until it curdles.

However, the milk may also be preripened in a preripening tank by the souring culture until a pH of 5.7 is reached. It is then pumped into the cheese vat with the addition of the desired amount of rennet. The vats contain 5000 to 12000 L of milk. They are provided with cutting and stirring devices and also with heatable jackets. In the pretreatment of milk up to the stage of the coagulation of the casein, any introduction of gas bubbles whether by entrainment during pumping or stirring or through gas-forming microorganisms must be avoided, since the production of curds containing bubbles of gas make processing difficult and cause losses of yield.

Cutting and stirring—The solid curd with a pH of 4.7, corresponding to 0.4 to 0.5% of lactic acid, is cut into pieces with an edge length of 1 to 2 cm. Slow stirring prevents the

pieces of curd from coalescing again and promotes the release of whey. Pronounced turbulence in the vat which leads to the abrasion of cheese dust from the pieces of curd is avoided because of the loss of yield.

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                        ┌──────────────────────┐
                        │ Pasteurized skim milk │
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                                              ─────Starter
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┌──────────────────┐   ┌───────────────────────────┐
│ Mixing in a trough │   │ Mixing in the preripening │
└──────────────────┘   │   tank, souring to pH 5.7 │
                        └───────────────────────────┘
                                                        Rennet
                        ┌──────────────────────┐
                        │  Souring, curdling    │
                        └──────────────────────┘
                        ┌──────────────────────┐
                        │      Cutting          │
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                        │ Stirring and standing │
                        └──────────────────────┘
                        ┌──────────────────────┐
                        │      Scalding         │
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                        ┌──────────────────────┐
                        │  Removal of whey      │ → Whey
                        └──────────────────────┘
Wash water and   →      ┌──────────────────────┐
  ice water             │ Washing and cooling   │ → Whey and water
                        └──────────────────────┘
                        ┌──────────────────────┐
                        │   Cheese curd         │
                        └──────────────────────┘
Cream ──────────→
Common salt ──────→
Spices ──────────→
Fruit preparations ─→
                        ┌──────────────────────┐
                        │      Mixing           │
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                        ┌──────────────────────┐
                        │      Filling          │
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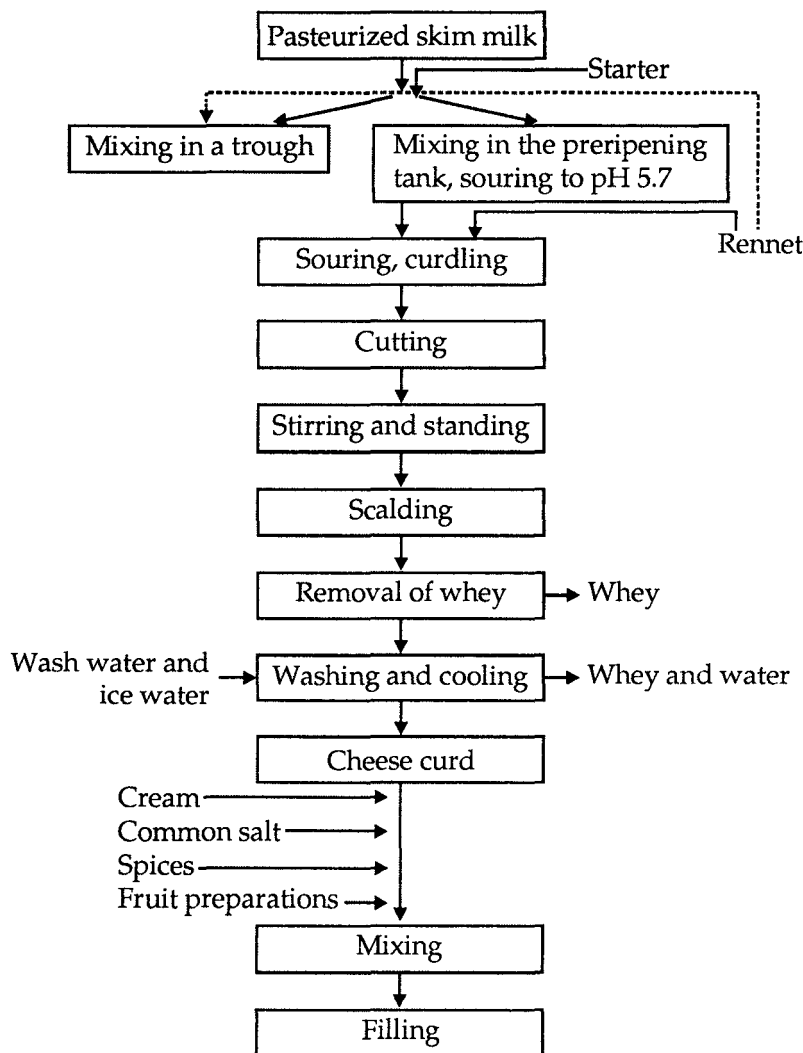
Fig. 13.2 : Manufacture of cottage cheese (Niederauer, 1978).

Scalding—About 30 minutes after cutting, the contents of the vat are heated quite slowly to temperatures of 42 to 60°C by blowing hot water or steam into the jacket of the vat with simultaneous stirring and are left under these conditions for sometime. This promotes the uniform release of whey from the grains of curd, so that a firm elastic curd arises with no formation of skin. This process lasts 1 to 3 hours.

Washing—After this, part of the whey is run off and, with stirring, the grain is washed with cold germ-free water and finally with ice water, which cools it to 8°C and the water is run off.

Mixing—The curd is mixed with cream, if desired with the addition of common salt. Spices or fruit preparations may also be added. After this, the granular fresh cheese is filled into packages.

Composition of cottage cheese according to Niederauer (1978):

Water	78.5%
Protein	12.3%
Fat	4.3%
Lactose	3.3%
Lactic acid	0.4%

Granular fresh cheese, especially when it contains flavouring additives, should be stored at 5°C in order to retain its organoleptic quality (Dolle, 1977: Diessel, 1976; Klein *et al.*, 1980; Niederauer, 1980).

13.6 HARZER CHEESE

Manufacture (Fig. 13.3)

This was originally manufactured from pure sour-milk quark, but today it is also made from quark that has been obtained by a mixed rennet-lactic acid coagulation process. The lactic acid fermentation can be carried out with mesophilic cultures *(Streptococcus lactis* and S. *cremoris)* or with thermophilic cultures *(Lactobacillus helveticus,* etc.). The dry-matter content should amount to at least 32%.
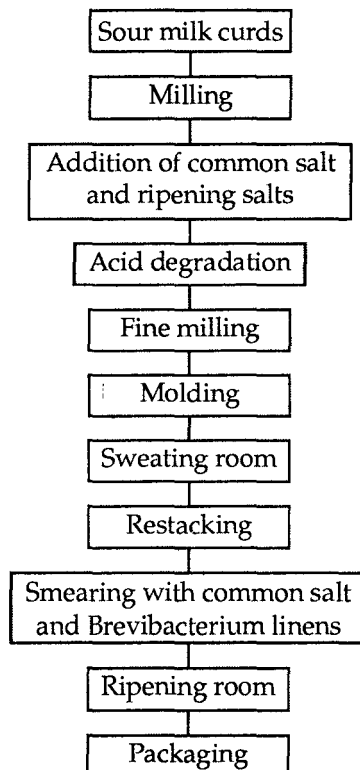
Fig. 13.3 : Manufacture of Harzer cheese.

Quark from various productions and manufacturers is milled to walnut size and mixed with 3 to 4% of common salt and 0.5 to 1.5% of ripening salts ($NaHCO_3$, $CaCO_3$). This is intended to neutralize part of the lactic acid in order to give a pH of 4.8 to 4.9. A further decomposition of the acids by yeasts is achieved by storage at room temperature for several hours to one day. After fine comminution, the curd is shaped in special machines. The freshly formed cheeses are incubated on boards in the "sweating room" at 20 to 25°C in 95% atmospheric humidity for 2 to 3 days. During this process, the surface of the cheese is deacidified by film-forming yeasts. After the formation of a light yellow smooth skin the cheeses are coated on all sides with a solution of 50 to 75g of common salt and 50 to 100 mL of a culture of *Brevibacterium linens* in a litre of water. After drying overnight, the cheeses are transferred to the ripening room where, at 12°C and a relative humidty of 90 to 95% the typical red streak is formed. The cheeses can then be packaged and market.

13.7 EMMENTAL (SWISS) CHEESE

Manufacture (Fig. 13.4)

Emmental cheese is produced from raw milk mainly in Switzerland, Germany, Finland, France, and Austria. There is a classical procedure of manufacture in the cheese vat in which 1000 L of milk gives one cheese. In addition, however, there are already many large-scale manufactures in which up to 12000 L of milk are processed in each cheese vat. The evening milk preripened overnight (12°C) is mixed with the fresh morning milk. The added starter culture consists of 1 to 2% of a culture of thermophilic streptococci (S. *thermophilus)* and lactobacilli (e.g., *L. helveticus)* in whey. In addition a few drops of a culture of propionic acid bacteria must be added. The curdling time with rennet, is about 30 minutes. The cutting of the curd and preliminary cheese formation require 45 min. The mixture of curd and whey is heated with stirring to 53°C within 30 to 40 min and is stirred for another 30 to 60 min. After the treatment, the grains of curd are about 2 to 3 mm in size. After the settling of the curd, it is taken up with a cloth and placed in molds. At first it is compressed under a load of 500 kg for 20 min, and then under 1000 kg for 60 min and finally under 1200 kg. During this procedure it must be turned several times. After overnight pressing, by the next morning the lactose should have been
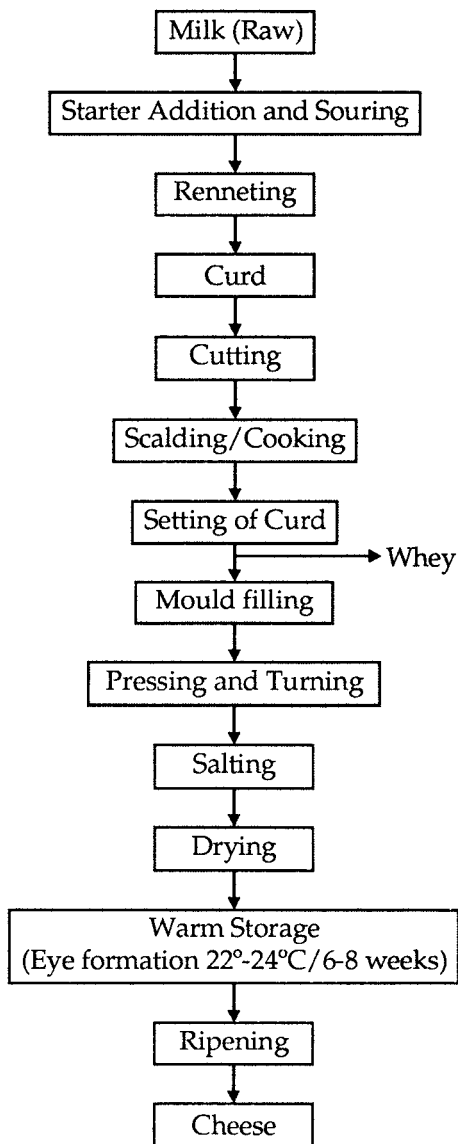
Fig. 13.4 : Flow diagram for manufacture of *Emmental* (Swiss) cheese.

fermented quantitatively to lactic acid. The pH of the cheese is then about 5.25. After this, the cheeses are placed in a salt bath for 2 to 3 days at 15°C. After drying in the salting room, which requires about 10 to 14 days, the cheeses are stored in the warm room for 6 to 8 weeks. At temperatures of 22 to 24°C, the lactic acid formed is fermented by the propionic acid bacteria to carbon dioxide and acetic and propionic acids, which gives rise to the characteristic formation of "eyes" and to the development of the taste of the Swiss cheese. After the conclusion of gas formation, the cheeses are ripened in the storage cellar at 12 to 14°C. During this time, the surface of the cheeses must be freed from the fungal coating that forms by washing. The optimum time of ripening is 6 months.

13.8 CHEDDAR CHEESE

Manufacture (Fig. 13.5)

Cheddar cheese is the preferred cheese product of the Anglo-Saxon countries (United Kingdom, USA, New Zealand).

The milk for its manufacture is heated at 72.2°for 15 sec and cooled to 31°C and is then treated with 1.5% of a single or multistrain starter. 21.8 mL of rennet per 100 kg of milk is stirred in mechanically in the vat. The curdling time amounts to 30 to 35 min. After this, the curd is cut into cubes with a size of about 1 cm^3. Five minutes after the end of this process and within less than 30 minutes the material is heated (scalded) to 38 to 39°C. The mixture is stirred at this temperature to an acidity in the whey of 0.15% of lactic acid (about 6.7 °SH*) (about 2.5 h). The curd is allowed to settle and the whey is run off through filters. A rake-type stirrer is then drawn through the mass of curd several times during a period of 20 min in order to achieve the desired grain size. During this process the acidity rises to about 0.23 to 0.24% of lactic acid. The mass of curd is now heaped up in order to achieve its coalescence ("cheddaring"). After this heaping
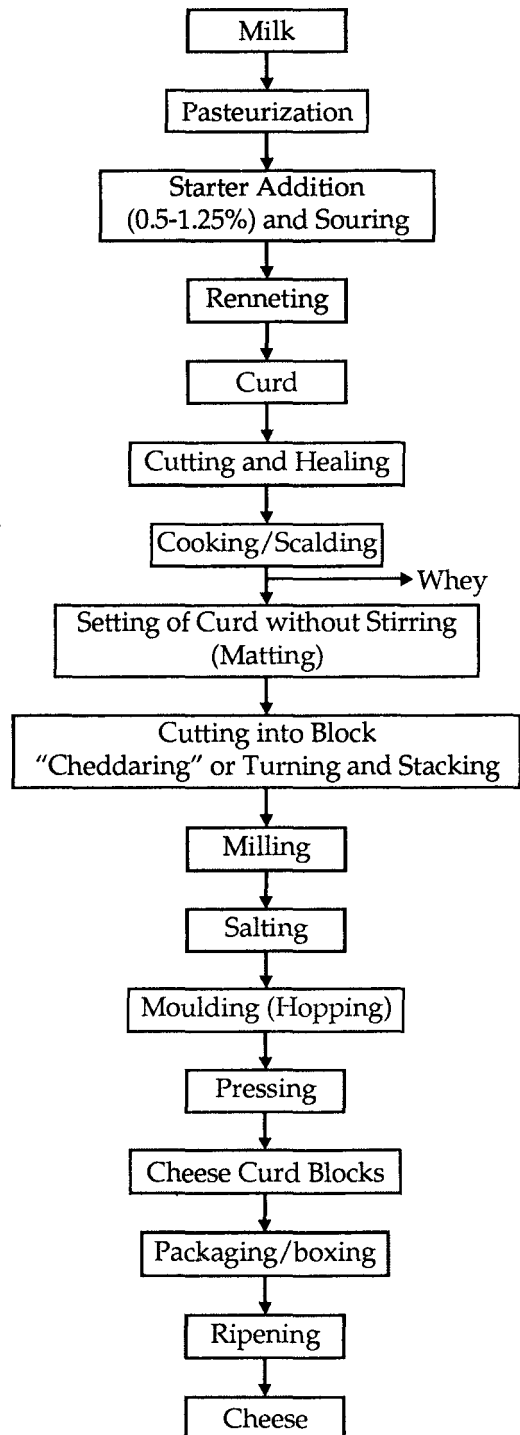
Fig. 13.5 : Flow chart for manufacture of cheddar cheese.

procedure, pieces weighing 8 to 12 kg are cut out from this mass of curd and are placed in layers above one another. During this period of storage of about 2 to 2.5 h, the lactic acid content rises to 0.5 to 0.6% with a simultaneous drainage of whey. The cheddared blocks are then milled to form shreds of curd and are salted (about 3 kg of salt/100 kg of cheese mass). The salted mass is filled into molds and pressed. After this, the cheeses are taken from the molds and coated with plastic foil or hot wax. In this form they are placed in the ripening cellar. They remain there at 7 to 10°C under a relative humidity of 55% for 1 to 12 months. Cheddar cheese can be coloured by the addition of dyestuffs (usually carotenoids).

13.9 LIMBURG CHEESE

Manufacture (Fig. 13.6)

The pasteurized kettle milk is treated with 0.2 to 0.5% of mesophilic starter. Then 15 to 20 mL of liquid rennet (1:10000) is added per 100 L of kettle milk. The temperature at adding the rennet is between 29 and 35°C, depending on the fat content. The whole curdling time amounts to 40 to 45 minutes. The curd is cut into 1.5- to 2-cm pieces. After 10 mm, it is cut again and after 15 min some whey is taken off and the coagulum is agitated for 5 to 10 min in order to make it somewhat firmer. The coagulum is charged into molds, and after about 1 hour it is turned in the molds. Limburg cheese is then kept for 24 h in the salt bath (16 to 18% NaCl between 16 and 18°C). After the salt bath, the cheese is allowed to drain and dry out. It is dried for another 2 to 3 days in the ripening cellar. Then it is greased, i.e., coated with a red smear culture (*Brevibacterium linens*). Greasing must be repeated three to eight times, depending on the size of the cheese in order to obtain a thick surface growth particulary when the cellars are relatively dry and have somewhat high temperatures, since otherwise the growth of moulds takes place. Good ripening conditions are 14°C and a
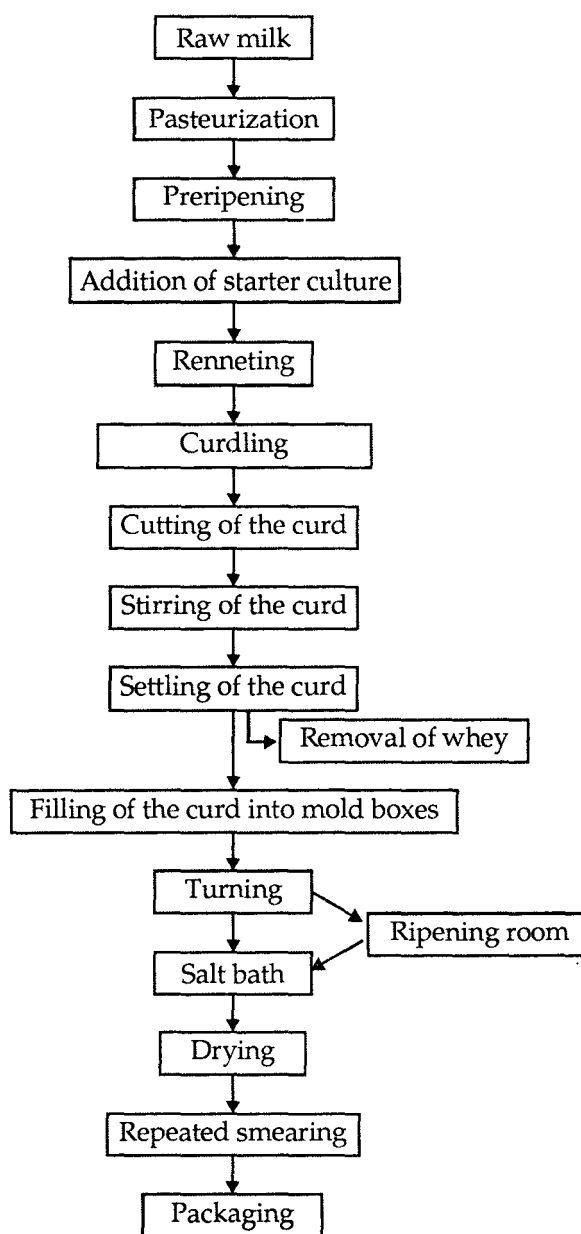
Fig. 13.6 : Manufacture of Limburg cheese.

relative humidity of 90 to 95°C. After 3 to 4 weeks or, in the smaller cheeses, after only 2 to 3 weeks, as soon as the red smear has developed well, the cheeses are packed. While in the classical cheese dairy the greasing of the surface was carried out by hand, today this task is performed mechanically.

13.10 CAMEMBERT CHEESE

Manufacture (Fig. 13.7)

Pasteurized milk is inoculated with 1 to 2% of starter. Today, the addition of suspension of spores of *Penicillium caseicolum* or *P. camemberti* is also carried out before the addition of rennet, as a rule. The renneting temperature is 35 to 36°C and the amount of rennet 20 to 25 mL (1:10000) per 100 L of kettle milk. The curdling time is 15 to 20 min. This may be followed by a thickening time of 40 to 50 min. The curd is carefully cut into cubes with a size of 1.5 to 2 cm. From cutting to the removal of the curd takes about 25 min. The curd is filled into suitable molds. After having stood for 10 minutes, the cheeses are turned, and the process repeated two or three times in the course of a day. In order to permit souring to take place during this period, the temperature of the room should be about 20 to 24°C. Before the cheese is immersed in the salt bath, the pH should have fallen to about 4.8 to 8. Depending on the fat content and the size of the cheeses, they are left in the salt bath (18 to 20% NaCl, 16 to 20°C) for between 40 and 220 min. The salt bath itself should be slightly acidic (about 20 to 40 °SH). The cheeses are transferred from the salt bath racks to the ripening racks. They are first stored in the drying room for 2 to 3 days (19°C, then falling to 17°C). In the ripening room a temperature of 16°C then falling to 14°C is possible. The relative, humidity in the drying room should be 70 to 80%, and in the ripening room 85 to 95%. The cheeses are turned fairly frequently in the ripening and drying rooms. The growth of mold appears after about 3 to 4 days. After 9 to 11 days, the cheese can be packed in foil and marketed. Camembert is one of the varieties of cheese which, because of the
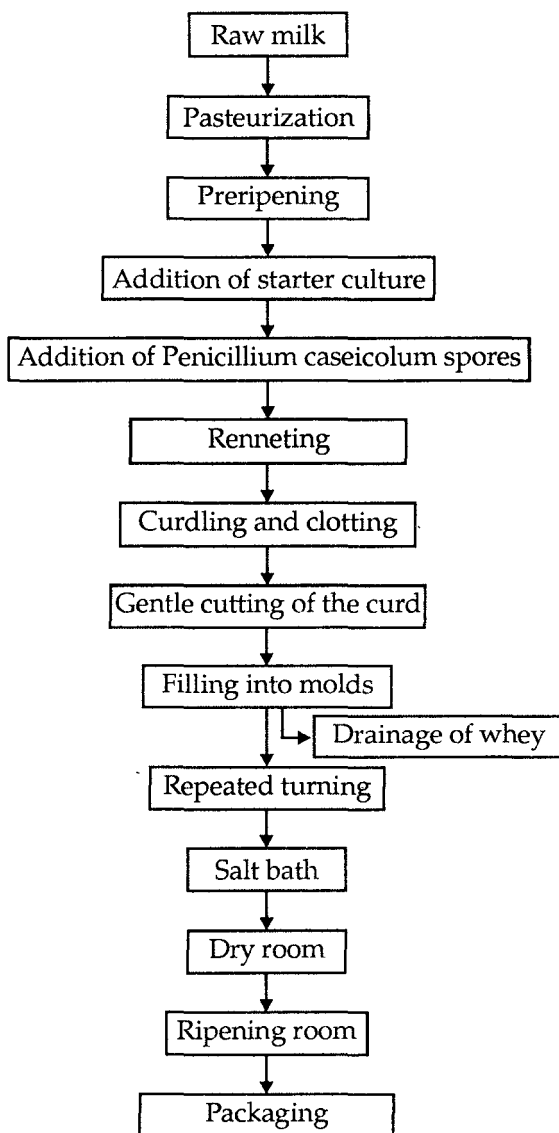
Fig. 13.7 : Manufacture of Camembert.

growth of molds rapidly ripens or changes its consistency at a suitable storage temperature after the customer has acquired it. The proteolytic enzymes of the Camembert mould then lead to a softening of the cheese mass, coupled with a liberation of aroma products and, finally, ammonia through the action of deaminating enzymes. This has the consequence that contaminating microorganisms such as coliform bacteria that may have passed into the cheese during its production can develop well in the Camembert.

13.11 ROQUEFORT CHEESE (BLUE CHEESE)

Manufacture (Fig. 13.8)

Roquefort cheese is produced from fresh raw ewe's milk. The average protein content is 6.5% and the average fat content 8%. As a rule, whey from the cheese production of the previous day, which contains a mixed culture of lactic acid streptococci and lactic acid bacilli, is used as the starter culture. The temperature for the growth of this culture is 35°C. *Penicillium roqueforti* is added to the kettle milk in the form of a suspension of spores. The fungus can also be cultivated on bread and then be dusted on to the cheese curd with the dry spores during the shaping process. The renneting temperature is 30°C and the curdling time 120 to 150 min with 25 mL of rennet (1:10000) to 100 L of kettle milk. The curd is cut into 1- to 3-cm cubes. During the next few hours, the curd is stirred a few times. Then it is allowed to settle and the whey is
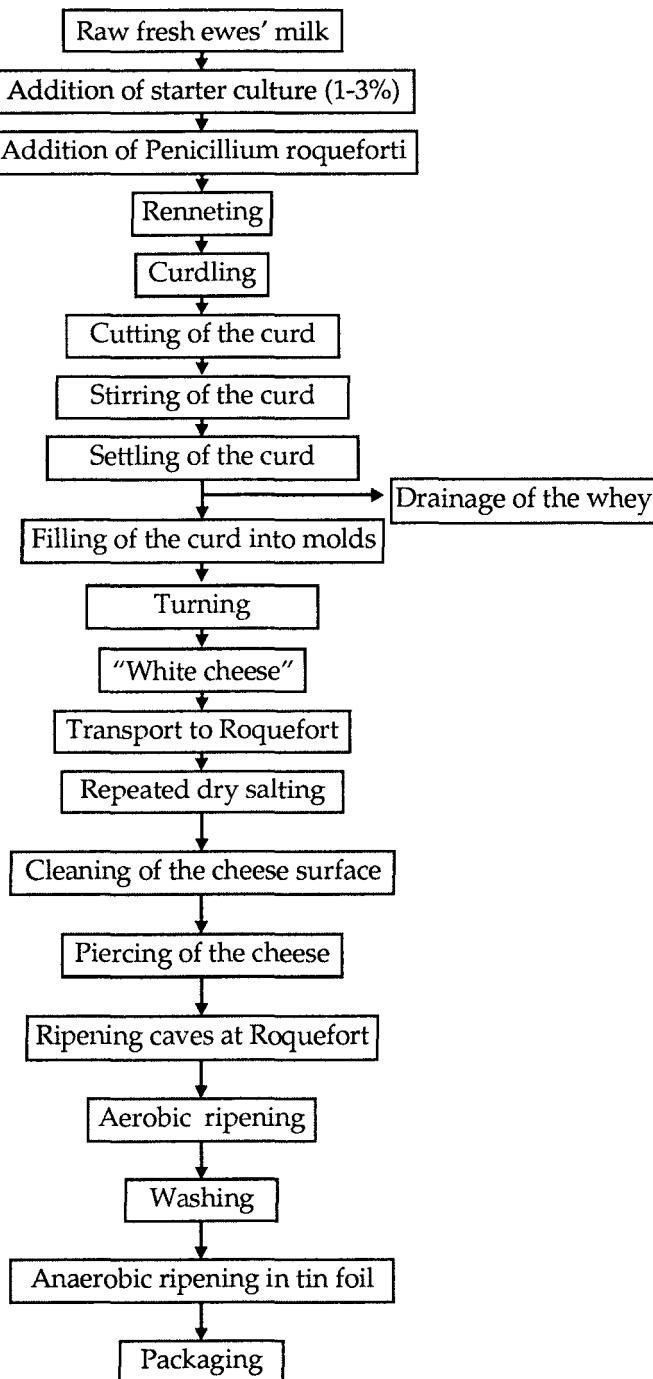
Fig. 13.8 : Manufacture of Roquefort.

drawn off. The coagulum is filled into suitable stoneware or tinplate molds (diameter 20 cm, height 10 cm). The drainage of the whey in the molds lasts about four days. They are turned once a day. During this phase a slight heterofermentation of the lactic acid type takes place in the coagulum which serves to create cavities for the development of the fungus. These still soft cheeses are transported (from as far away as Corsica and the Pyrenees) to Roquefort. They are salted by hand, with coarse sea salt in salting rooms (temperature 9 to 10°C) (several times in the course of 3 to 5 days). The salt-tolerant microorganisms that develop are removed from the surface by brushes. Then the cheeses are manually or mechanically pierced with needles in order to create access to the mass of cheese for the oxygen necessary for the growth of the fungus. Ripening must take place exclusively in the natural cellars at Roquefort which run for several kilometers through the mountain. The temperature in these cellars is constant at 7 to 10°C and the atmospheric humidity is between 90 and 100%. At first, the cheeses are stored in layers on edge at 8 to 10°C and a relative humidity of 96% for 18 to 23 days. Yeasts and bacteria that develop on the surface of the cheeses are scraped off. After the end of this aerobic ripening, to induce the anaerobic phase the cheeses are packed into tinfoil and are stored at 7 to 8°C, again on edge. Because of the lack of oxygen the growth of the fungus slows down. Ripening, proteolysis, and lipolysis proceed further under the action of the proteolytic and lipolytic enzymes that have already been secreted in the cheese dough. This anaerobic phase lasts at least three months. A ripened cheese can be stored at 1°C for another 5 to 10 months. After the conclusion of ripening, the cheeses are taken from the tin foil and are packed in aluminium foils or plastic bags. The high quality blue cheeses known in other European countries, such as Danish Blue, Bavaria Blu, and Bresse Bleu, are cheeses made from cows' milk which, however, is otherwise treated as in the manner of Roquefort. The speciality "Bavaria Blu" is a cheese in the interior of which the Roquefort fungus has grown but which has on the surface the white *Penicillium caseicolum*. It is therefore a true white-blue cheese.

13.12 DUTCH CHEESE (EDAM AND GOUDA)

A group of cheeses originating in Holland are sometimes referred to as Dutch cheeses. The best known and popular of these are Edam and Gouda. Both are semisoft to hard cheeses having a characteristic mild flavour that varies in intensity with age. The basic manufacturing procedure is similar to Cheddar with modifications to obtain a higher moisture content and the curd is not generally salted prior to hooping. The Edam cheese is made into round ball-shaped cheeses weighing from about ½-4½ lb in the United States and is covered with a red wax.

Gouda is made into the shape of a flattened sphere and may weigh from less than 1 lb to 50 lb. Most common are the smaller sizes of less than 1 lb and having the red wax coating similar to Edam.

Flow diagram for production of Edam and Gouda cheese are explained in Fig. 13.9.

13.13 MOZZARELLA CHEESE (PASTA FILATA TYPE)

Another Pasta Filata type cheese is Mozzarella, a soft, plastic curd cheese originating in southern Italy. The cheese was originally made from buffalo milk. Mozzarella is made by methods similar to that used for Provolone but modified to produce a cheese containing higher moisture. It is common practice to produce the curd at one facility and, after draining

off the whey, shipping the curd to a dealer who completes the manufacturing process when he is ready to market the cheese. He completes the manufacture by heating the curd in hot water and stretching and molding it into irregular spherical shapes weighing approximately ½-1 lb. The cheese is eaten fresh with little or no ripening.

Scamorza is another Pasta Filata cheese, originating in Italy and is similar in characteristics to Mozzarella and is made by a similar process. Mozzarella and Scamorza are both eaten fresh and are used extensively in cooking.

Flow diagram for production of Mozarella cheese is explained in Fig. 13.10.

13.14 PASTEURIZED PROCESS CHEESE/CHEESE SPREAD

The second broad classification of cheeses is the category known as process cheeses. Pasteurized process cheese is prepared by comminuting and mixing, with the aid of heat, one or more cheeses of the same or two or more varieties of cheese, and certain emulsifying salts. Water, seasoning, and colour may be added. Cheddar cheese is the variety most commonly processed but Swiss, Limburger, Brick, and other similar varieties are also used. Process cheese has certain characteristics which make it popular. It can be attractively packaged and is less subject to spoilage and shrinkage in weight than natural cheese. To the consumer, it has a more uniform flavour, has no waste, and has excellent keeping quality.

When certain optional dairy ingredients such as cream, skim milk, whey, or their solids are added to the cheese blend and processed, the product is called a pasteurized process cheese food or a pasteurized process cheese spread. Both products may contain less fat and more moisture than process cheese as prescribed in the Federal Definitions and Standards of Identity. In addition, the pasteurized process cheese is spreadable at 21°C.
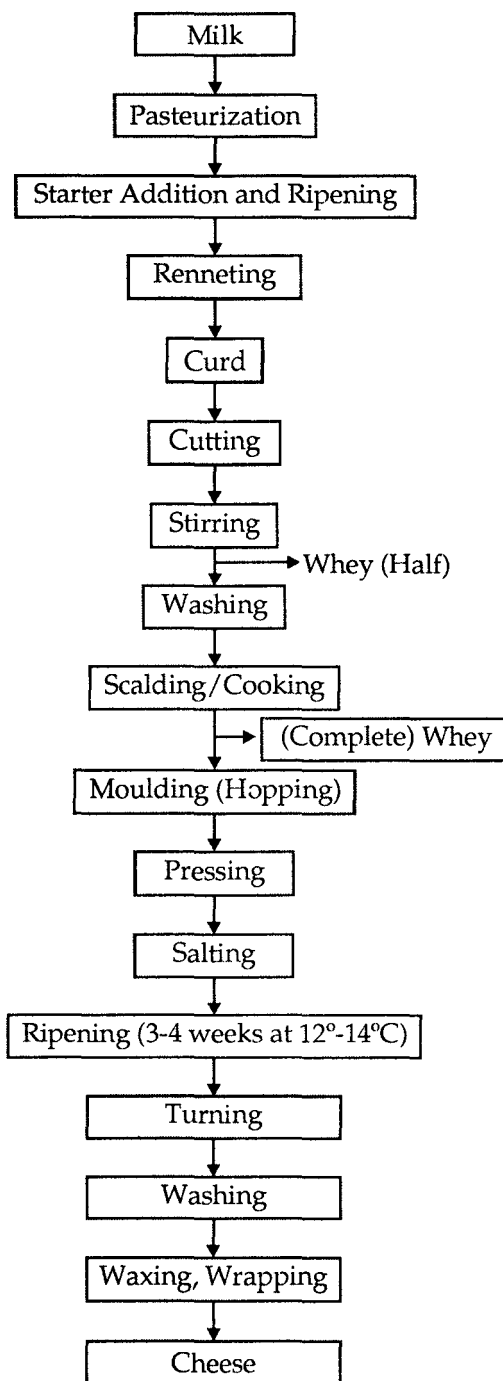
Fig. 13.9 : Flow diagram for production of Edam and Gouda cheese.

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┌─────────────────────────────────────────────┐
│ Milk-Buffalo's or Cow's or a Mixture of Both │
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                        ▼
              ┌──────────────────┐
              │  Pasteurization  │
              └──────────────────┘
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┌─────────────────────────────────────────────┐
│       Starter Addition (0.05-0.5%)           │
│ and Ripening (To 0.02% developed acidity)    │
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                        ▼
              ┌──────────────────┐
              │     Renneting    │
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                 ┌─────────────┐
                 │    Curd     │
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                ┌──────────────┐
                │   Cutting    │
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                │   Stirring   │
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                │   Whey-Off   │
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            │    Curd Ripening   │
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│ Stretching in Hot (82°C) Water (Pulling the Curd) │
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               ┌──────────────┐
               │   Moulding   │
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              │    Cheese      │
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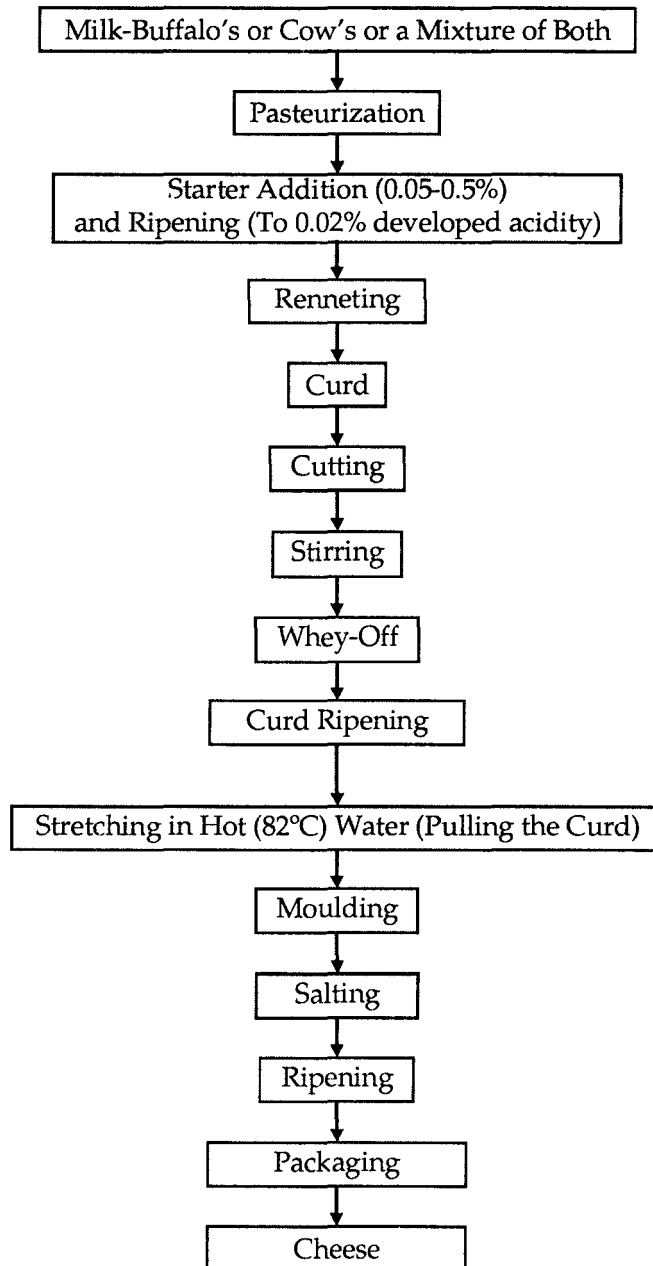
Fig. 13.10 : Flow diagram for manufacture of Mozzarella cheese.

In India, most of the processed cheese is made from cheddar cheese. In a mixture of cheese, appropriate quantities of emulsifier, whey powder, salt, flavouring agent, colour, water etc. may be added and heated to 80-85°C for 4-8 min. (*processed cheese*) or 85-95°C for 8-15 min (*cheese spread*) and filled directly into retail containers, usually tins or tetrapacks.

Flow diagram for manufacture of processed cheese is explained in Fig. 13.11.

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         ┌─────────────────────┐
         │  Natural Cheese(s)   │
         └─────────────────────┘
                   ↓
┌──────────────────────────────────────────────┐
│ Analysis and Selection of Cheese of Proper Age Group │
└──────────────────────────────────────────────┘
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      ┌───────────────────────────┐
      │  Tempering and Cleaning    │
      └───────────────────────────┘
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     ┌─────────────────────────────┐
     │ Quartering (Dividing into 4 parts │
     │        and Grinding)         │
     └─────────────────────────────┘
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   ┌────────────────────────────────────┐
   │            Additives               │
   │ (Water, Colour, Salt and Emulsifier) │
   └────────────────────────────────────┘
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      ┌───────────────────────────┐
      │   Heating and Stirring     │
      └───────────────────────────┘
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      ┌───────────────────────────┐
      │         Packaging          │
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      │          Cheese            │
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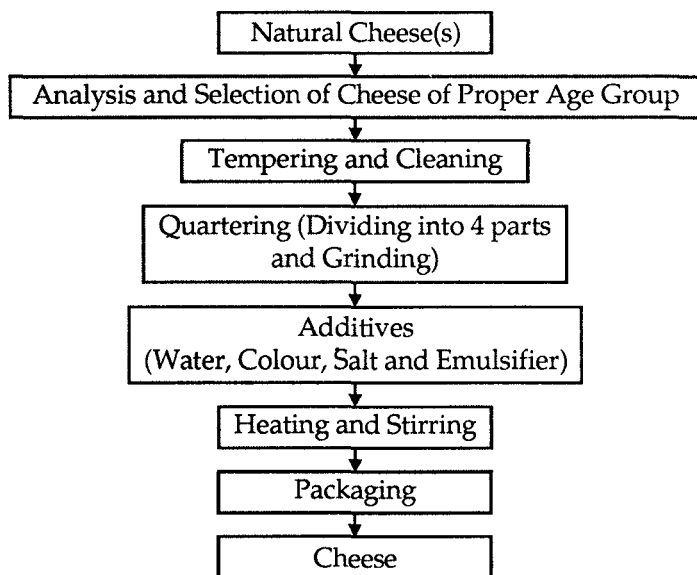
Fig. 13.11 : Flow diagram for manufacture of processed cheese.

13.15 MICROBIOLOGICAL TESTS AND STANDARDS FOR CHEESE IN INDIA

The microbiology of cheese is complex and involves a number of microorganisms. When monitoring a ripening process, selective enumeration of different groups of bacteria like acid producers, proteolytic, lipolytic, gas producers, etc. can be done.

However, for judging the quality of final product, test for contaminants or pathogens can be done. These tests include: (1) Coliform count, (2) Yeast and mold count, (3) Test for anaerobes-Clostridia, (4) Detection of *Staph. aureus* and other pathogens. The microbiological standards for cheese are not available in India, except coliform count in cheese powder (See Table 13.1).

Table 13.1 : BIS recommended standards for cheese in India

Sr. No.	Characteristic	Natural cheese (Hard)	Processed cheese	Processed cheese spread	Soft Cheese Milk	Skim	Cheese powder
1.	Moisture % by mass, maximum	43	47	60	48	48	05
2.	Milk fat (dry basis) % by mass, minimum	42	40	40	50	13 (Max.)	40
3.	Salt (added NaCl) % by mass, maximum	03	03	03	Optional	Optional	5.5
4.	Coliform count per gram, maximum	-	-	-	-	-	10

□□□

14

ICE CREAM AND RELATED
FROZEN DAIRY DESSERTS

14.1 INTRODUCTION

Frozen desserts, known to the Romans, and consumed by French and English Royalties in the 15th century, came to the American scene in the first half of the 18th century. Developments in dairy technology and merchandising resulted in the production of more than 763 million gallons of ice cream plus an additional 380 million gallons of related products. The fact that 9.4% of the total milk produced in the United States is used in frozen desserts compared to 17.7% for cheese making indicates the economic importance of the ice cream industry. Ice cream is a very popular dairy product all over the world.

The ice cream is nutritionally rich and can protect the microorganisms when present. The microorganisms in ice cream can come from the ingredients used in its manufacture or are added during processing steps. This chapter discusses manufacturing of ice cream and related frozen desserts and various microbial aspects involved in it.

14.2 DEFINITIONS

Ice cream, a frozen food product prepared from a mixture of dairy ingredients, sweeteners, stabilizers, emulsifiers, and flavouring materials, is the most important of the frozen desserts. Dietary considerations have, in recent years, prompted consumers to use substantial quantities of ice milk, a product similar to ice cream but containing less milk-fat. Other major related products include sherbets (usually a tart product made from the same basic ingredients as ice cream but fortified with fruit and/or fruit juice) and ices (prepared from water, sugar, fruit juices, stabilizer, acid, and colour). Products in which a vegetable or animal fat is substituted for butterfat are similar to ice cream, ice milk, or sherbet and are classified as mellorine-type frozen desserts. Roughly ice cream contains about 50%

of air in it, but mix intended for freezing contains on an average of 10% fat, 11% SNF, 14% sugar and 0.5% stabilizer, emulsifier, flavour and colour.

14.3 COMPOSITION AND INGREDIENTS

The basic composition of ice cream, ice milk, sherbet, and water ice is fairly uniform due to Federal Standards and State laws regulating the minimum and sometimes maximum percentages of the different ingredients. Although the individual States have their own legal standards, the differences are only minor, and in time most State laws will conform to the Federal Standards. Table 14.1 lists the minimum and maximum levels of the different constituents of frozen desserts as permitted by the Federal Frozen Dessert Standard (Code of Federal Regulation, 21 CFR Part 20).

Table 14.1 : Minimum standards for frozen dairy desserts

| | Ice Cream[1] | | Ice Milk[2] | Fruit Sherbet[3] | Water Ice |
	Plain	Bulky Flavours			
Milk-fat					
min (%)	10.0	8.0	2.0	1.0	—
max (%)	—	—	7.0	2.0	—
Milk solids					
min (%)	20.0	16.0	11.0	5.0	—
max (%)	—	—	—	5.0	—
Food solids					
min (lb/gal.)	1.60	1.60	1.30	—	
Weight					
min (lb/gal.)	4.50	4.50	4.50	6.0	—
Stabilizer					
max (%)	0.50	0.50	0.50	0.50	0.50
Emulsifier					
max (%)	0.30	0.30	0.30	0.30	—
Salts					
max (%)	0.24	0.24	0.24	—	—
Acidity					
min (%)	—	—	—	0.35	0.35

[1] Frozen Custard—Same standards as for ice cream, except plain custard shall have a minimum egg yolk solids content of 1.4% and 1.12% for bulky-flavoured frozen custard.

[2] No permitted reduction in standards for bulky-flavoured ice milk.

[3] No minimum total foods solids provided. Non-acid sherbet does not require the 0.35% acidity.

The manufacturer can choose from a wide choice of ingredients as specified by the Federal Definitions and Standards for Frozen Desserts. These optional ingredients are

grouped as follows: (a) dairy ingredients, (b) sweetening ingredients, (c) flavour ingredients, (d) other optional ingredients, (e) stabilizers and emulsifiers, (f) mineral salts, and (g) colouring. Quality ice cream depends, first, on the selections of good ingredients and, second, on a well-balanced mix. Representative formulas for the various frozen desserts are given in Table : 14.2.

Table 14.2 : Representative frozen dessert formulas[1]

	Ice Cream					
	Premium[2] (%)	Average (%)	Ice Milk (%)	Sherbet (%)	Ice (%)	Soft-Serve (%)
Milk fat[3]	16.0	10.5	3.0	1.5	—	6.0
Milk solids not fat	9.0	11.0	12.0	3.5	—	12.0
Sucrose	16.0	12.5	12.0	19.0	23.0	9.0
Corn syrup solids	—	5.5	7.0	9.0	7.0	6.0
Stabilizer[4]	0.1	0.3	0.3	0.5	0.3	0.3
Emulsifier[4]	—	0.1	0.15	—	—	0.2
Total solids	41.1	39.9	34.45	33.5	30.3	33.5
Pounds/Gallon of mix	9.17	9.36	9.46	9.48	9.4	9.30
Draw From Freezer						
Overrun (%)	65-70	95-100	90-95	50	10	40
Approx lb/gal. finished product	5.4	4.6	4.8	6.25	8.5	6.5

1. Frozen desserts containing vegetable fat (mellorine type) are permitted in some states. A wide variation of composition exists depending on individual State Standards.
2. If classified as Custard or French, it must contain not less than 1.4% egg yolk solids.
3. Milkfat content regulated by individual State.
4. Usage level as recommended by manufacturer of stabilizer and emulsifier.

14.4 FUNCTIONS AND SOURCES OF INGREDIENTS

Milk fat increases richness and creamy flavour and produces a characteristic smooth texture and full body in ice cream; it is, therefore, the most important ingredient. The limitations, however, include its relatively high cost as well as the relatively small amount which can be consumed because of its high caloric value and sustaining power. The usual sources of milk fat are fresh cream, whole milk, frozen cream, plastic cream, and butter.

The milk solids not fat (MSNF) are high in food Value and relatively inexpensive. Their influence, on flavour is indirect by rounding out the flavour of the finished product. The protein part of the MSNF improves texture, gives body to the frozen products, and is essential for the incorporation of stable air cells. The lactose adds to the sweetness which is, however, derived mainly from sucrose or corn syrup solids. The percentage of lactose limits the total MSNF which can be used. When the free water contains more than 8.7% lactose, the lactose might crystallize and produce a "sandy" ice cream. The usage level of

MSNF lies between 9 and 13% dependent upon the product composition. The most common sources for MSNF used in frozen desserts are fresh skim milk, condensed skim milk, non fat dry milk powder, and condensed whey or whey powder. Legally as well as functionally whey solids may only replace 25% of the MSNF. Sweet cream buttermilk finds only a limited use in ice cream but has special value because it contains phospholipids (as an emulsifier).

Sweeteners provide the necessary sweetness, enhance the flavour, and produce a desirable body and texture. They depress the freezing point which, up to a point, is desirable. Sucrose is the most widely-accepted source of sugar. Corn sweeteners can be used to provide up to 40-50% of the total sweetness.

The corn sweeteners (dextrose, corn syrup solids, and corn syrup) are significantly lower in sweetness than sucrose. However, the advantages of their use includes increasing solids without excessive sweetness, imparting a "chewiness" to the body of ice cream, and providing added protection against development of coarse texture in ice cream upon storage.

Stabilizers and emulsifiers are added to the frozen dessert mixes in rather small quantities for a specific purpose. It should, however, first be pointed out that the other ice cream ingredients also possess stabilizing and emulsifying properties. Furthermore, the mechanical processing of the mix has a definite effect upon stabilization and emulsification. Stabilizers function in ice cream through their ability to form gel structures in a water solution or to combine with water as water of hydration. Stabilizers bind water and, by virtue of this function, produce smoothness in body and texture by reducing ice crystal size and retarding ice crystal growth during freezing and storage. They provide uniformity and chewiness of product and resistance to melting. Gelatin was for many years the most widely-used stabilizer. At the present time, many other materials are used; mostly colloids derived from seaweeds or plants; sodium carbomethylcellulose may also be used. Each stabilizer has its advantages and disadvantages and often a combination of two or more is used.

Emulsifiers function in ice cream through their ability to reduce the interfacial tension between the fat-aqueous phases of the emulsion. This results in a finer dispersion of the fat in ice cream and a fine air cell structure, producing a stiff and dry body character as well as an improved apparent richness, and that the rate of agglomeration and coalescence of the fat globules involves not only the emulsifier but also a number of other factors important in affecting ice cream quality. The emulsifiers can be divided into the following groups:

(1) Glyceryl or sorbitan compounds of fatty acids, large stearates.

(2) Polyoxyethylene derivates of fatty acids, or their glyceryl or sorbitan compounds.

The dairy ingredients which contain natural emulsifying constituents include: milk proteins, lecithin, phosphates, and citrates. Egg yolk produce are high in lecithin and have long been used in ice cream.

A number of **mineral salts** are listed under the optional non-dairy ingredients which may be used in frozen desserts. Their main function has to do with the heat stability of the milk protein and they may influence some characteristics of the finished product.

Air and water in frozen desserts are necessary ingredients. The air, incorporated as very tiny air cells, and the water, partly frozen into small ice crystals, give the product the palatability, the texture, and body necessary for good quality and pleasant eating characteristics. Both ingredients are important for the physico-chemical system which ice cream represents.

The increase in product volume resulting from air incorporation is referred to as overrun and is defined as the volume of ice cream obtained in excess of the volume of mix.

The "**percentage overrun**" is calculated as follows:

$$\text{Overrun} = \frac{\text{volume of ice cream} - \text{volume of the mix}}{\text{volume of the mix}} \times 100$$

The Federal Standards or State laws on the weight per gallon of finished product determine the maximum overrun percentage. The usual range varies from 40% for the hand-filled "bulk" package to 75-100% for machine-filled product.

14.5 PROCESSING AND MANUFACTURING

Processing of the ice cream mix consists of selecting the composition of the mix; calculating and blending the different ingredients; and pasteurizing, homogenizing, and cooling the mix.

The pasteurization of the mix provides a safeguard for the health of the public since undesirable bacteria are destroyed during this process. Pasteurization is universally required by States and cities; however, the temperature and time combination may differ.

The most common temperature and time systems for pasteurization are:

Method	Temp (°C)	Time
Batch system	68	30 min
High temperature- short time ·	79	25-30 sec
Ultra high temperature	99-130	0-4 sec
Vacuum pasteurizer	90-97	2 sec

All commercial mixes are homogenized; a process which consists of passing the mix under pressure through a small opening, resulting in a reduction in size of the fat globules to at least 4.0 m in diameter, with the vast majority smaller than 2.0 m. The mix is homogenized at a temperature in the range of 49°-74°C and at a pressure between 1500 and 3000 psi. The most important advantages of homogenization are uniform and permanent suspension of fat globules, smoother texture, improved whipping ability, and diminished danger of churning during freezing.

With the use of improved stabilizers and freezing techniques, storage of white mix for 3-6 hr and of chocolate mix for 12 hr is sufficient to improve freezing; thus making unnecessary the longer time of "aging" the mix earlier thought necessary to permit hydration and phase equilibria of mix constituents.

14.6 FREEZING PROCESS

The freezing process consists of a rapid withdrawing of heat from the ice cream mix, thus freezing part of the water into ice crystals and the concurrent incorporation of air under agitation. To produce a smooth quality ice cream, it is essential that the ice crystals and air cells be small; this is accomplished by rapid withdrawal of heat and vigorous agitation. One can use a formula to calculate the percentage of water frozen as ice at any given temperature. The amount of water frozen as ice in the freezer varies between 30 and 60%, depending on drawing temperature and composition of the mix. The size of the ice crystals frozen during mechanical agitation of the mix and the distribution of the water in the unfrozen portion of the mix determine, in part, the smoothness of the finished product.

The second important function of the freezing process is the incorporation of air. Whipping properties of the mix are dependent to a large extent upon its composition, although this is of less importance when the continuous freezer is used. The amount of air incorporated into the mix and the size of the air cells influence the body and texture of the resulting ice cream.

Batch, continuous, and soft-serve freezers are the primary machines used for manufacturing frozen desserts. The batch freezer consists of a cylinder mounted vertically or horizontally. A motor operates a dasher inside the cylinder. The dasher is equipped with scrapers for removing the ice cream as it freezes to the inside of the cylinder, and it serves as a beater, facilitating the incorporation of air. The refrigerant (brine, ammonia, or freon) circulates through the double wall of the freezer cylinder which consists of a steel or copper tube with an inner lining of stainless steel. The mix enters the freezer at approximately 4°C.

As the refrigerant is applied, the temperature drops rapidly to –6°C and the flow of refrigerant is discontinued when the mix develops a rather firm body. Operation of the dasher and attached mechanism is continued until the desired overrun (incorporation of air) is obtained. The minimum temperature reached by the ice cream (in the batch freezer) is about –4°C. On completion of freezing, the batch of ice cream, as a semi-frozen mass, is drawn from the batch freezer into containers and placed in the hardening room.

The continuous freezers, with capacities ranging from 80 to 1000 gal. per hr, have replaced the batch freezer in commercial plants. Ice cream mix and air are pumped into the freezer chamber and continuously agitated by a dasher. A refrigerant cools the inner surface of the chamber where the dasher knives scrape the semi-frozen ice cream from the wall while the unfrozen mix takes its place. The Vogt and the Creamery Package continuous freezers are well known and are similar in their operation. In the Vogt freezer, the air is metered into the mix prior to entering the freezing chamber; while in the Creamery Package freezer, air is injected directly into the chamber. An ice cream pump in the latter case moves the semi-frozen ice cream from the chamber.

The low-temperature continuous freezer is capable of delivering the semi-frozen product at –8°C compared to –5°C for conventional freezers. This lower temperature is accomplished by a second freezer chamber which has an eccentrical dasher. The finished product has more resistance to adverse handling conditions.

The soft-serve freezer is a combination of a batch and a continuous freezer. After an initial filling of the freezing chamber, mix is automatically fed from a container into the chamber whenever product is withdrawn. The product is kept chilled in the chamber when no soft-serve product is withdrawn.

In the manufacturing of fruit, nut, or candy ice cream when visible particles are desired, the flavour, fruit juices, and colour are added to the mix but the fruit, nuts, and candy must be incorporated into the product after it leaves the freezer via a fruit feeder.

Variegated flavours, often called "royaled" or "rippled" ice cream, are very popular. These ice creams are made by injecting one or more fudge or fruit bases into the ice cream as it leaves the freezer. A number of variegator machines are available which can be controlled to inject the desired amount of the flavour base. Some of the most popular variegated flavours are chocolate, caramel, butterscotch, strawberry, raspberry, and orange.

The final step in ice cream manufacturing is the hardening process. When ice cream is drawn from the freezers, it has a semi-fluid consistency and is not stiff enough to hold its

shape. It is, therefore, hardened until it reaches a temperature of –18°C or below. Here, as in the freezer, fast hardening will facilitate the formation of small ice crystals. The hardening takes place in rooms where the temperature is –29° to –35°C or in specially designed hardening tunnels where air at a temperature of –50°C is blown on packages of incoming ice cream. Numerous types of fast hardening devices are in general use for wrapped units, including plate or contact hardeners, blast tunnels, and roller beds. Ice cream may be hardened within 4 hr in any of these systems. In a modern ice cream plant the processing and manufacturing have become mechanized and automated. A description of such an automated plant is described by Arbuckle in *Ice Cream*.

14.7 FLAVOURS OF FROZEN DESSERTS

Ice cream owes its variety and popular appeal to many pleasing flavouring materials which can be used in its manufacture. The flavours are added directly into the mix when powders, liquids, or purées are used. With fruits or nuts, a fruit feeder is used which incorporates the particles into the semi-frozen ice cream as it leaves the freezer. Although there are over 150 different ice cream flavours, a survey showed that the following flavours account for more than 80%: vanilla 47.5, chocolate 14.0, variegated chocolate 7.3, nut meats 7.10, strawberry 5.5, coffee 1.5 and peach 1.5 percent. The Federal Standards of Identity specify the minimum flavour as well as labeling requirement.

Vanilla ice cream is flavoured with an extract of the vanilla bean, or combinations of natural vanilla extracts and vanillins. In some areas, ground vanilla beans are added for eye appeal.

Fruits, fruit concentrates and essences are quite extensively used as flavourings in frozen desserts. Fresh frozen fruit is preferred; strawberries, raspberries, pine-apple, peaches, banana, and cherries are the most favoured. The amount to be added varies according to the particular fruit, from 25% for pineapple to 30% for peaches, calculated on the weight of the finished product.

The most important nut flavours include buttered pecans, buttered almonds, cashews, walnuts, and pistachios. The amount of nuts added to the mix may vary from 5 to 10 lb per 100 lb of mix. Increasingly popular are the variegated ice creams, where 10-15% of a prepared sauce is injected usually into vanilla or chocolate ice cream. The most popular flavours in this category are: chocolate, butterscotch, caramel, marshmailow, and several fruit sauces.

14.8 NOVELTIES AND SPECIALITIES

Novelty ice creams or frozen products come in many flavours, shapes, sizes, and colours. They are individually wrapped and intended for individual servings. The annual production is estimated at close to 10 billion pieces and accounts of or approximately 25% of the industry's total gallonage. The most popular novelty items are: chocolate or candy-coated ice cream and ice milk bars usually with a stick, Eskimo Pies (chocolate or candy-coated ice cream bars), ice cream sandwiches (ice cream slice between two wafers), ice cream cups (plain or with a fruit or candy topping), and sherbet and ice-like mixtures frozen on a stick.

Soft ice cream and ice milk have the same ingredients as regular ice cream and ice milk. The major difference between the two is that soft ice cream is dispensed to the consumer in a semi-frozen state drawn directly from the freezer and contains from 30 to 50% overrun.

14.9 STRUCTURE OF ICE CREAM

The internal structure of ice cream is determined by the composition of the mix, the manufacturing procedure, and by the distribution and particle size of the components as they occur in the frozen ice cream. The ingredients in the frozen ice cream are present in a complicated physicochemical system. A large portion of the water is frozen and the resulting ice crystal size greatly affects the crystalline structure of the ice cream. Air cells of different sizes are dispersed throughout the entire structure. Fat globules are in emulsion; milk proteins, insoluble salts, and stabilizers are present as particles of colloid dimensions; the sugars and soluble salts are in true solutions. The size, shape, arrangement, and distribution of all these components determine the internal structure of the ice cream.

The texture of ice cream is directly related to the structure—the size, shape, number, and arrangement of the air cells, ice crystals, lactose crystals and fat clumps, and the thickness of the foam lamellae. In a quality product a smooth texture is indicative of uniformly small ice crystals and air cells, and no detectable lactose crystals. A coarse and icy texture is caused by relatively large ice crystals, while sandiness is the result of lactose crystallization. A buttery or greasy texture is caused by the presence of butterfat lumps. A snowy texture is the result of large and/or too many air cells.

The body in ice cream is related to the mass of ice cream as a whole and refers to its consistency or firmness, and resistance to melting. An ice cream with a heavy or soggy body has too little air. Too much air results in a fluffy body.

14.10 DEFECTS OF ICE CREAM

Flavour, body, and texture are the principal factors used in judging the quality of the finished ice cream. Flavour defects are imparted by the mix ingredients (oxidized, acid, and cooked; or by the flavouring materials—too strong, unnatural, etc.). Body detects are commonly described as crumbly, soggy, and weak; while the common texture defects are coarse, icy, fluffy, sandy, and buttery. Another defect to which consumers object is a too high or too pale colour.

Two defects, although important but not readily noticeable, are bacterial defects and melting defects. States and communities have imposed maximum permissible numbers of bacteria in the product for public health reasons. The most common defects in meltdown characteristics are curdled meltdown, whey-like consistency upon melting, and ice cream which "does not melt."

An unclassified defect is "shrinkage." When this defect occurs, ice cream, after hardening, shrinks away from the top and sides of the packages and gives a "not full" appearance.

14.11 SHERBETS AND ICES

Sherbets and ices are manufactured in a manner similar to ice cream. One of the important factors in making these products involves a well-balanced mix and the choice of

stabilizer, since product quality depends on the proper functioning of the stabilizer to give a smooth texture and a firm body and to aid in controlling overrun. Only stabilizers which control the overrun, prevent syrup drainage, and eliminate crumbly body should be selected. One or a combination of the following stabilizers can be used: gelatin, gum tragacanth, pectin, cellulose gum, or algin products.

The kind and amount of sugars used in sherbets and ices are important in relation to properties of the finished product. The use of dextrose or corn syrup to the extent of 25-30% of the amount of sucrose, is advisable as a means of preventing surface crustation.

Tartness differentiates sherbets and ices from ice cream. The amount of acid needed varies with the amount of sugar and the amount of fruit acid contained in the fruits or fruit combinations. The legal minimum is 0.35% calculated as lactic acid.

Citric acid is the most commonly-added acidulant. The more common flavours for ices and sherbets are orange, pineapple, raspberry, lime, and lemon. The amount of fruit required varies with the intensity of flavour and the quality of fruit; it should be from 13 to 20% of the weight of the finished product, except for lemon and lime where 7% is usually sufficient. Recently, "non-acid" sherbets have been permitted; however, the legal standard is the same as for regular sherbets except for acidity.

14.12 OTHER FROZEN DESSERTS

There are a number of other frozen desserts which are usually classed with the ices and sherbets.

(1) **Granites** are made from the same mixes as ices, the main difference being that they are frozen so as to permit little or no whipping. The product is, therefore, hard, has a low overrun and a coarse texture, resulting from a minimum of stirring during freezing.

(2) **Punches** are essentially ices with alcoholic beverages used in place of, or supplementary to, fruit juices. Rum flavouring or cordials may be used in place of the alcoholic beverages. An ice or sherbet in frozen condition is sometimes added to the fluid punch.

(3) **Frappes** are made in identically the same manner as sherbets, differing only in that they are served at the consistency of sherbets when the latter leave the freezer. A frappé is, accordingly, a sherbet before it goes to the hardening room.

(4) **Soufflés** are made from the same mixes as sherbets, but with chilled and beaten whole egg added at the freezer at the rate of about two eggs per gallon of mix. A further difference is that soufflés are usually frozen with a high overrun to obtain a fluffy product.

(5) **Bisque** ice cream is usually higher in fat than regular ice cream and contains a bread or confection product such as dried macaroons, marshmallows, or sponge cake.

(6) **Parfait** is also high in fat, containing egg yolks with or without nuts or fruits, or other natural flavours.

(7) **Mousse** is a frozen whipped cream to which sugar and flavour have been added. Ice cream mix is also usually added to the cream before whipping and freezing.

(8) **Ice cream pudding** is a high fat ice cream with nuts and fruits, highly flavoured or seasoned. Common types of puddings are Manhattan, Nesselrode, plum, and Oriental.

(9) **Aufait** is a brick ice cream, consisting of layers of one or more kinds of ice cream, alternating with layers of frozen fruit.

(10) **Lacto** is a frozen product manufactured from cultured skim or whole milk, eggs, and sugar, with or without natural flavouring.

The old-fashioned "home-made" type of ice creams are now commercially produced. These products have a very low overrun and are extremely cold to the taste.

14.13 NUTRITIVE VALUE OF FROZEN DESSERT

Frozen desserts are excellent sources for food energy because of their high sugar content. Furthermore, ice cream and ice milk are excellent food products containing all and often more of the milk solids than milk itself. The digestibility and palatability of these products add to their nutritive value. Table : 14.3 shows the nutrient content of frozen desserts.

Table 14.3 : Nutrient composition per 100 grams of various frozen desserts

	Vanilla Ice Cream		Vanilla Ice Milk	Orange Sherbet	Orange Ice
Fat (gm)	10.2	12.1	3.1	1.1	Trace
Protein (gm)	3.5	3.8	3.9	1.4	0.1
Carbohydrates (gm)	24.3	24.8	25.7	29.8	35.4
Total Solids (gm)	39.3	42.0	34.1	33.6	37.0
Calcium (mg)	135.0	144.0	150.0	59.0	1.0
Phosphorus (mg)	107.0	115.0	119.0	42.0	2.0
Sodium (mg)	73.0	78.0	82.0	33.0	Trace
Potassium (mg)	216.0	232.0	241.0	71.0	15.0
Iron (mg)	0.1	0.1	0.1	Trace	—
Magnesium (mg)	15.0	16.0	17.0	6	—
Vitamin A (IU)	412.0	486.0	124.0	44.0	14.0
Thiamin (mg)	0.04	0.05	0.05	0.01	0.01
Riboflavin (mg)	0.22	0.24	0.25	0.07	Trace
Niacin (mg Equiv)	0.96	1.02	1.06	0.37	0.02
Vitamin B_6 (mg)	0.06	0.07	0.07	0.02	—
Vitamin B_{12} (mg)	0.58	0.63	0.65	0.25	—
Vitamin C (mg)	—	—	—	—	—
Vitamin D (IU)	—	—	—	—	—
Vitamin E (IU)	0.3	0.3	0.1	Trace	—
Calories	204.0	223.0	146.0	135.0	142.0

14.14 EFFECT OF RAW MATERIALS ON MICROBIAL CONTENT

Good quality product can only be made from good quality raw materials. Ice cream may be prepared with several dairy and non-dairy ingredients. Hence, it may get a variety

of microorganisms. Milk and cream should be free from coliforms, off flavours and other spoilage agents.

(1) Skim milk powder used in ice cream may contain *B. cereus* and *Staph. aureus,* which can cause food poisoning.

(2) Butter or butter oil, when used, should be of high microbiological quality and in particular absence of *Ps. fragi* should be verified. It should also be checked for yeast and molds, coliforms and lipolytic bacteria.

(3) Sugar can be a major source of yeasts, molds and bacterial spores. It is available to use the sugar having mesophilic count of less than 20 and yeast and mold count of less than 1 per gram.

(4) Stabilizers and emulsifiers, rarely present any problem.

(5) In certain varieties, where fruits, nuts, chocolate, etc. are used, extreme care is necessary. Fresh and frozen fruits may contain yeasts, while nuts may be infected by molds. Coconuts have been occasionally implicated in Salmonellae infection.

(6) Packing materials will be good source of molds and bacterial spores. It should be effectively sanitized before use.

(7) After packing, the ice cream is transferred in hardening rooms where all the moisture goes in frozen state. Thereby, organisms do not get free moisture and hence cannot proliferate. However, they can survive for long periods.

In the entire manufacture process, contamination by human handlers, especially with pathogens, is also of great concern.

14.15 PUBLIC HEALTH SIGNIFICANCE OF ICE CREAM

Cases of diseases out-breaks through consumption of commercially manufactured ice cream are rare. However, when mix is not properly pasteurized or gets contaminated through processing plant, or humans, it can act as a vehicle of disease transfer.

Few cases of food poisoning, typhoid, dysentery, and abdominal pains have been reported caused by *Staph. aureus, Salmonella* spp., *Shigella sonnei* and *B. cereus* respectively. Pathogens once entered, can survive for long times in ice cream, e.g., *Sal. typhosa* can survive for 20 months at –20°C or *Brucella* spp. and paratyphoid organisms can remain viable for more than 4 years at –23°C.

The bacterial content of ice cream depends largely upon (1) the number present in the milk or cream at the time of preparation and (2) the number present in the various ingredients employed in its manufacture.

High total counts usually indicate neglect and unsanitary conditions. These may result from (1) poor quality of ingredients used, (2) improper pasteurization, (3) contamination after pasteurization, (4) improper aging, (5) unsanitary equipment, and (6) negligent and untrained personnel.

Organisms which may be found in ice cream include coliform bacteria, Micrococci, streptococci, spore-forming rods, yeasts and molds. Some are of dairy origin; others are from the ingredients used in its manufacture.

Ice cream is stored at temperatures of –17.8 to –28.9°C. At these temperature, there is a gradual but slow decrease in the bacterial population. The lactic acid organisms, i.e.,

those responsible for the souring of cream, fail to multiply. The presence of disease-producing organisms is generally the result of using contaminated cream in its manufacture. Cold is not a sterilizing agent and should not be depended upon to destroy all disease organisms in ice cream.

14.16 MICROBIOLOGICAL TESTS AND STANDARDS FOR ICE CREAM

Common tests recommended for microbiological examination of ice cream are— (1) Total bacterial count, (2) Coliform count, (3) Yeast and mold count, (4) MBRT, (5) Psychrotrophs, (6) Staphylococci and (7) Salmonella count.

BIS has prescribed a standard plate count (SPC) of less than 2,50,000/gram and coliform count of less than 90/gram with negative phosphate test for ice cream.

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BUTTER AND RELATED PRODUCTS

15.1 INTRODUCTION

Butter is a plastic mass consisting mainly of milk fat which has been made from cream. Butter (or Makkhan) has been made from milk from earliest times. Ancient manuscripts indicate that butter was used as a food in India around 2000 BC, and it is mentioned in Genesis and other biblical writings. It seems probable that the practice of using butter for food was introduced into Western Europe via Scandinavia. Butter has long been an important article of commerce, and was being exported from Norway and Sweden as early as the 14th century. The keeping quality of butter compared to milk has been known since early times.

Originally, butter was produced directly from milk, and the method of churning usually involved a stationary churn with rotating agitators operated manually.

The development of the butter making industry dates from about 1850 when gravity setting of cream was practised, but the invention of the mechanical separator in 1877 heralded the real beginning of the butter factory. About 1890, pasteurization of cream for butter making was introduced with consequent improvements in keeping quality.

The continuous butter making method developed in 1940s, based on the Fritz principle, quickly gained wide acceptance in countries manufacturing unsalted or lightly salted butter. The continuous machine has now largely replaced the stainless steel churn in all major butter producing countries.

Worldwide consumption of butter and butter fat products is estimated at 2,420,000 tons for countries where data are available (Table 15.1). The world stocks of butter continue to rise, and butter is consequently being stored for increasing periods of time with additional demands on overall quality. Various aspects for production of butter and related products are discussed in this chapter.

Table 15.1 Total consumption (1000 tons) of butter and butter fat products (1993)

Country	1,000 tons
Austria	33.8
Australia	58.3
Belgium	70.0
Canada	84.776
Switzerland	37.5
Germany	555.9
Denmark	21.5
Estonia	8.91
Spain	9.0
Finland	27.0
France	389.8
United Kingdom	205
Hungary	9.7
India	58.51
Iceland	0.589
Italy	98
Japan	92
Netherlands	50.4
Norway	9.7
New Zealand	31.9
Sweden	19.9
United States	533
South Africa	14.748
Total	2,420

Source: Adapted from Bulletin of IDF No. 301, 1995, p. 13.

15.2 COMPOSITION AND TYPES OF BUTTER

Butter is a water-in-oil emulsion, wherein butterfat forms the continuous phase. This is in contrast to cream, which is an emulsion of fat globules suspended in an aqueous phase. Thus, an emulsion phase reversal occurs during the manufacture of butter. This happens in churning of cream, and, as a result, fat is concentrated in the product. Butter contains more than 80% fat (typically approximately 81%), 15% to 16% moisture, less than 0.5% of carbohydrates and protein, and 2% sodium chloride (if added). The pH of sweet cream (unfermented) butter is between 6.1 and 6.5 (Milner, 1995). Many countries allow sodium chloride and lactic cultures as the only non-milk additives in butter (Milner, 1995). Some countries allow neutralization of cream and addition of natural colouring agents (e.g., annato, carotene, and turmeric).

There are essentially two kinds of butter: sweet cream, which may or may not be salted, and ripened-cream butter. Citrate in cream is fermented by lactic acid bacteria in ripened-cream butters to produce acetoin and diacetyl; the latter imparts characteristic flavour to the product (Adams and Moss, 1995). Ripened-cream butters are more popular in continental Europe, whereas unripened or sweet-cream butter is preferred in the United States, Ireland, England, Australia, and New Zealand (Adams and Moss, 1995).

Milk fat recovered from whey produced during cheese making can be passed through a separator to produce whey cream. This whey cream can be processed into butter, which is often indistinguishable from butter made from sweet cream (Halpin-Dohnalek and Marth, 1989b). Whey cream butter can be manufactured from neutralized or non-neutralized salted or unsalted whey cream.

15.3 MANUFACTURE OF BUTTER

The manufacture of butter (Fig. 15.1) is characterized by the following three processes:

1. **Concentration of the fat phase of milk**—This is done by milk *separation* and *standardization* of resultant cream to the desired fat content.

2. **Crystallization of the fat phase**—Large numbers of small solid fat crystals are required. *Pasteurization* temperature applied to cream yields fully liquefied butterfat. Thus, *cooling* (greater than 4 hours at approximately 5°C) is necessary to develop an extensive network of stable fat crystals. In ripened-cream butter, addition of lactic acid bacteria to pasteurized cream cooled to 16°C to 21°C is followed by incubation until a pH near 5 is obtained. Cooling to 3°C to 5°C is begun after the product reaches approximately pH 5.

3. **Phase separation and formation of a plasticized water-in-oil emulsion**—*Churning* and *working* break the oil-in-water (o/w) emulsion and result in a plasticized water-in-oil (w/o) emulsion. These processes occur in both batch and continuous churns. During churning, violent agitation is used to disrupt membranes on milk fat globules (Brunner, 1976). Effective clumping of fat globules to form a continuous matrix requires selection of an optimal temperature, usually 5°C to 7°C, for a batch churn. The optimal temperature is dependent on triglyceride composition and hence the physical state of the fat (Brunner, 1976). Churning is ineffective if all fat is either liquid or solid. The proper blend of liquid and solid fat is necessary. Continuous churn operations require temperatures that account for the fat content of cream and hardness of the butterfat. The optimum temperature for churning is often selected empirically by operators to minimize fat losses (Varnam and Sutherland, 1994).

After churning and working, the butter is *washed* and *salted.* Salting is done after washing to prevent loss of salt. *Packaging* occurs after salting and may be done directly into retail portions or in bulk containers (25 kg is common) (Varnam and Sutherland, 1994). National intervention boards in the European Economic Community stipulate a *storage* temperature of −15°C; however, lower temperatures are frequently used. A temperature of −30°C was effective for storing butter in excess of 1 year (Varnam and Sutherland, 1994). Stored butter is then subjected to *repackaging* into retail containers. Butter from different manufacturers may be blended together during repackaging, and sometimes garlic, chopped herbs, and diacetyl concentrate are added for additional flavour (Varnam and Sutherland, 1994). Thus, butter manufacture involves separation of cream from raw milk, standardization to the desired fat content, pasteurization, possible addition of lactic acid bacteria (when

ripened-cream butter is manufactured), churning, working, washing, salting, packaging, storage, and repackaging (see Fig. 15.1). All of these activities affect the microflora of the final product.
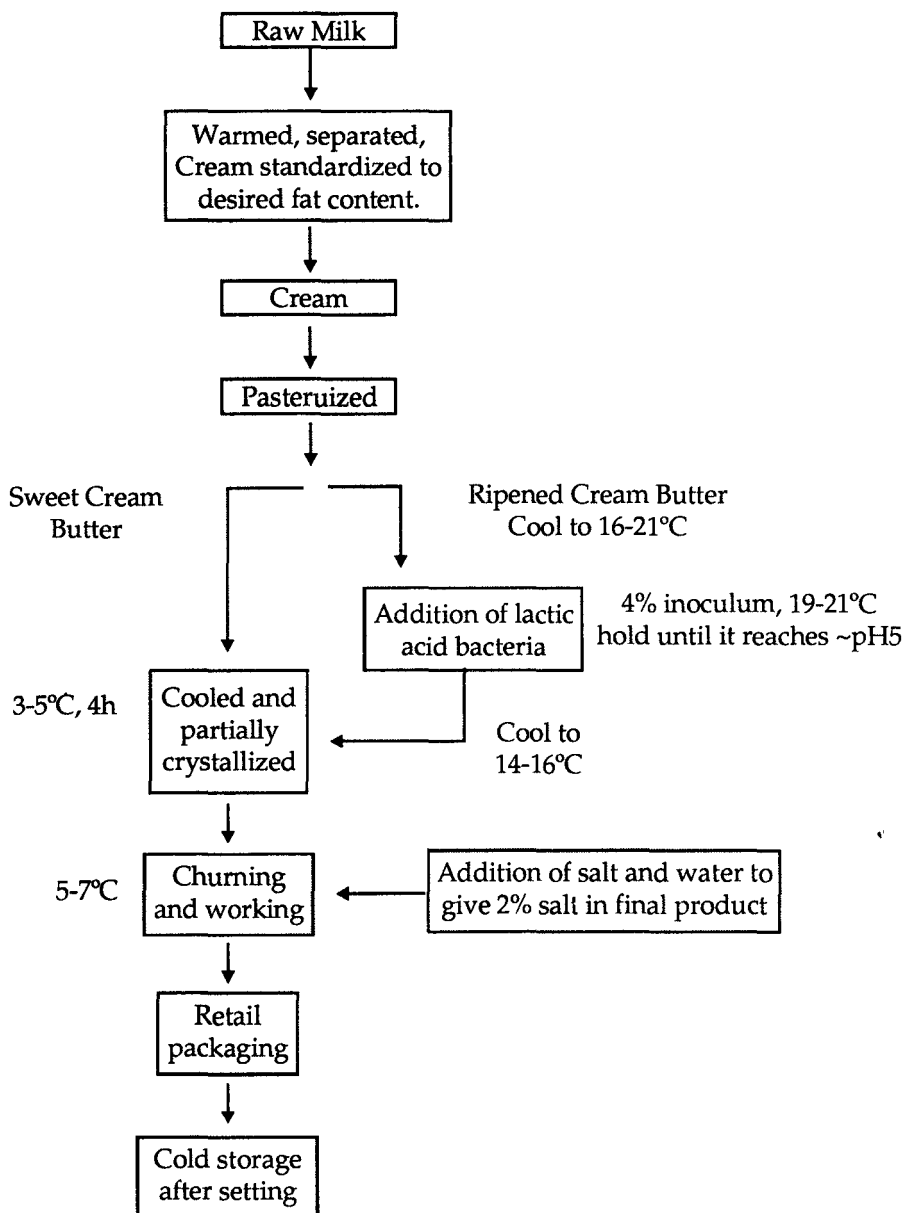
Fig. 15.1 : Production of butter (Adapted from Milner, 1995).

15.4 MICROBIOLOGICAL CONSIDERATIONS (BUTTER)

The microbiology of butter reflects the microflora present in cream from which it is made, fluid used to wash or salt the curd, sanitary conditions of process equipment,

manufacturing environment, process hurdles that limit microbial growth and survival, and conditions under which the product is stored.

15.4.1 Cream

The main source of microorganisms in butter made under excellent sanitary conditions is cream. Fat globules rising in milk carry microbes present in raw milk (Milner, 1995). Raw milk can be contaminated with a wide variety of microbial pathogens and spoilage microorganisms. The microflora of raw milk is related to that found in the cow's udder and on the hide and milking utensils and lines (Jay, 1992). Proper handling and storage conditions should result in a predominantly Gram-positive microflora in raw milk. Gram-negative microbes, along with yeasts and molds, are typically less heat resistant than Gram-positive microorganisms and are more likely to be destroyed by pasteurization (Jay, 1992). Psychrotrophic *Bacillus* and *Clostridium* spp. have been found in 25% to 35% and 8% of raw milk samples, respectively (Jay, 1992; IDF/FIL, 1994). These organisms would be expected to survive pasteurization of cream. Raw milk is occasionally contaminated with *Mycobacterium* spp. (Jay, 1992; IDF/FIL, 1994), and controversy exists over survival of *Mycobacterium paratuberculosis* (the cause of Johne's disease in cattle and putative agent of Crohn's disease in humans) during milk pasteurization (Chiodini and Herman-Taylor, 1993; Grant *et al.*, 1995, 1996; Keswani and Frank, 1996). A review of pathogenic microorganisms in raw milk was prepared by the International Dairy Federation (IDF/F1L, 1994).

15.4.2 Importance of Pasteurization

Butter was contaminated with *Streptococcus agalactiae*, *Streptococcus pyogenes*, and two strains of *Staphylococcus aureus* when made with unpasteurized milk from a cow whose quarters were artificially infected with these organisms. These bacteria persisted 6 months both in salted (2%) and unsalted sweet-cream butter and in ripened (6 and 12 days at 21°C) cream butter. *Brucella abortus* inoculated into unpasteurized raw cream also survived 4 months at approximately 7°C in salted and unsalted sweet cream butter. This organism also survived 3 months in unsalted and salted, ripened-cream (6 and 12 days at 21°C) butter. All storage was at approximately 7°C. Butters made from contaminated cream that was subsequently pasteurized at 62.8°C for 30 minutes did not contain these pathogens (Bryan and Bryan, 1944). Pasteurization of cream from raw milk is designed to eliminate vegetative microbial pathogens and reduce numbers of potential spoilage organisms. In the United States, cream must contain not less than 18% fat. Dairy ingredients with more than 10% fat must be pasteurized at temperatures 3°C higher than milk (e.g., 66°C for 30 minutes for cream versus 63°C for 30 minutes for milk) (Code of Federal Regulations, 1995). However, heat-resistant microbes such as some strains of *Lactobacillus*, *Enterococcus*, and spores of *Bacillus* and *Clostridium* will survive. Temperatures between 95°C and 112°C are commonly used to inactivate them (Schweizer, 1986). Cream is also heated to deactivate lipases (which cause hydrolytic rancidity in butter), reduce the intensity of undesirable flavours (e.g., from feed ingredients), activate sulphydryl compounds (which can reduce the auto-oxidation of butter), and liquefy fat for subsequent control of crystallization (Schweizer, 1986).

15.4.3 Ripening

Many people in western and northern Europe prefer the flavour of butter manufactured from microbiologically ripened cream (Pesonen, 1986). Traditionally,

pasteurized cream (after cooling to 6°C to 8°C for greater than 2 hours to initiate fat crystallization, followed by warming) at 19°C to 21°C is inoculated with lactic cultures composed of pure or mixed strains of *Lactococcus lactis* spp. *lactis*, *Lc. lactis* spp. *cremoris*, *Leuconostoc mesenteroides* ssp. *cremoris*, and *Lc. lactis* ssp. *lactis* biovar *diacetylactis*. Ripening occurs for 4 to 6 hours until a pH of 4.6 to 4.7 is achieved. The product is cooled to stop the fermentation. In this process, spoilage microorganisms are controlled primarily through the bacteriostatic effect of lactic acid produced by the starter culture.

15.4.4 NIZO Method

The NIZO method (Kimenai, 1986) for producing a cultured butter has been allowed in several countries and is used in many factories in western Europe. In the NIZO method, starter culture are not added to cream, but, instead, a mixture of diacetyl-rich permeate and starter cultures is worked into butter. Permeate is produced by fermentation of partly delactosed whey or other suitable media containing milk components by lactic acid bacteria (i.e., *Lactobacillus helveticus*). After incubation for 2 days at 37°C, the medium is subjected to ultrafiltration for removal of proteins and bacteria and further concentrated (Kimenai, 1986). During ultrafiltration, macromolecules are retained by a semi-permeable membrane and concentrated in a retentate, whereas low molecular weight solutes pass through the membrane in a permeate stream. The pH of butter made with this process is more easily adjusted in the desired range of 4.8 to 5.3. It has been claimed that this permeate can be stored at 4°C for more than 4 months (Kimenai, 1986). Advantages cited for this process include the following (Kimenai, 1986):

1. Greater control of the manufacturing process by dividing it into more independent steps (e.g., greater flexibility for choosing the most appropriate temperatures for both butter consistency and propagation of cultures)
2. Lower copper content of butter made by this method, hence, reducing the risk of oxidative defects
3. Lower free fatty acid content, hence, lessening the chance of hydrolytic rancidity (soapy flavour) of the final product
4. Less starter culture needed to be produced
5. Greater freedom to choose different types of starter cultures to better control butter flavour
6. Elimination of problems associated with ripened cream that may become too viscous to pump to the churn
7. A yield of sweet, not cultured, buttermilk
8. Greater quality of butter after 3 years of cold storage than that of traditionally manufactured butter stored 3 years

Homofermentative lactic acid bacteria such as *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* are used to produce lactic acid from lactose in dairy products. However, flavour production requires addition of a heterofermentative organism such as *L. mesenteroides* ssp. *cremoris* or *Lc. lactis* ssp. *lactis* biovar *diacetylactis* to produce diacetyl (Jay, 1992). Diacetyl, in addition to imparting flavour, inhibits Gram-negative bacteria and fungi (Jay, 1992).

15.4.5 Churning and Working

The bacterial load of buttermilk is typically greater than that of cream or butter (Milner, 1995). When culture-ripened cream is used to manufacture butter, most of the starter culture organisms are retained in the buttermilk; however, some remain in the butter. In several studies, butter made from cultured cream retained 0.5% to 2.0% of the culture present in the cream (Hammer and Babel, 1957). Olsen et al. (1988) found that numbers of *Listeria monocytogenes* were 6.7 to 15 times higher in pasteurized but subsequently inoculated creams than in butter manufactured from the same cream. In an earlier study (Minor and Marth, 1972), *Staphylococcus aureus* behaved similarly. These organisms are Gram-positive, and it is unclear how other microorganisms with different cell wall and membrane structures distribute themselves (e.g., *Mycobacterium* noted for its hydro-phobicity) between cream and butter. Diacetyl content of cream increases during churning; agitation during churning favours the oxidative processes needed for diacetyl production (Foster *et al.*, 1957). The pH of salted butter can prohibit formation of diacetyl (Foster *et al.*, 1957).

15.4.6 Moisture Distribution During Churning and Working

It has been estimated that 10 to 18 billion droplets of water are dispersed in 1 g of the w/o emulsion that is butter (Hammer and Babel, 1957). Given the low microbial load expected in pasteurized sweet cream (less than 20,000/mL) (Jay, 1992), most of the droplets are sterile. This depends on the degree of dispersion of droplets and the microbial level in the cream (Hammer and Babel, 1957). The diameter of water droplets in conventionally made butter has been reported at <1—>30 μm. Water droplets in butter manufactured by the Fritz, Alfa, and Cherry–Burrell continuous processes have been reported to be less than 1 to more than 15 μm, less than 1 to less than 7 μm, and less than 1 to 30 μm, respectively (Brunner, 1976).

Fritz-type Continuous churns are generally favoured worldwide; however, many phase-inversion types of churns that use highly concentrated cream (e.g., 80% "plastic" cream) are used in the former Soviet Union and other eastern European countries (Vamam and Sutherland, 1994).

The Fritz butter making process is based on the same principles as traditional batch churning. For example, crystallization of fat is carried out in cream with phase inversion and fat concentration during churning and draining. However, phase inversion methods rely on concentration of cream to approximately 80% in a centrifugal separator, followed by phase inversion, cooling, and then crystallization of fat (Munro, 1986). Several modifications of this approach have been developed, including the Alfa and Cherry-Burrell processes mentioned earlier.

The Meleshin phase inversion process and modifications of it are in wide use in eastern Europe and the former Soviet Union (Munro, 1986). In the Meleshin process, pasteurized cream is concentrated to a fat content slightly above that of the final product (e.g., 61% to 85%). It is then standardized and cooled to 11°C to 13°C in a series of scraped surface heat exchangers, each called a "transmutator," wherein phase inversion and crystallization occur (Munro, 1986). Varnam and Sutherland (1994), Kimenai (1986), and Munro (1986) have provided more detailed descriptions of continuous butter manufacturing processes.

The number of water droplets greater than 30 µm in diameter is inversely proportional to the time of working during conventional (batch churn) butter manufacture (Hammer and Babel, 1957). A consequence of uneven distribution of droplets containing microorganisms is a high degree of non-homogeneity regarding microbial distribution in butter. Thus, testing butter for quantitative microbial quality should be based on a three-class attribute plan (Smittle, 1992) to account for this variability. Inadequate working of the butter results in less dispersion of water droplets and promotes microbial spoilage (Hammer and Babel, 1957; Foster et al., 1957). Greater microbial growth was observed in serum when it was separated from unsalted butter than in butter before separation of serum. However, separation of serum from salted butter increased the rate of microbial destruction in the serum (Hammer and Babel, 1957, reporting on Hammer and Hussong, 1930). Thus, nutrients and salt in separated serum were more available for growth or inhibition of microorganisms, respectively, than when butter was in the normal physical state. This implies that the availability of nutrients or inhibitors is limited by the fine dispersion of water droplets (Foster et al., 1957). Droplet size is ideally less than 10 µm (Varnum and Sutherland, 1994).

15.4.7 Washing and Salting

Butter granules may be rinsed to remove excess buttermilk (Foster et al., 1957); however, this is not often done. If butter is washed, then salting is done after washing to prevent loss of salt. Salt added to butter can be expected to inhibit microbial growth. Salt must be distributed evenly in the moisture phase of the product to effectively inhibit microbial growth in contaminated water droplets. However, working does not result in a homogeneous distribution of salt in the water droplets (Hammer and Babel, 1957; Mimer, 1995). Salting creates an osmotic gradient between salt granules and buttermilk. This tends to cause aggregation of water droplets and can lead to free moisture and a defect called "mottling". Adequate working and use of finely ground salt can minimize this defect.

Use of brine is restricted to products with less than 1% salt because the brine cannot contain more than 26% salt (w/w). Sometimes, slurries of salt in saturated brine solutions containing up to 70% sodium chloride are used. Salt granules used for production of a slurry should be less than 50 µm in diameter. The salt slurry should also be of high purity, with less than 1 mg lead, 10 mg iron and 2 mg copper per litre (Varnam and Sutherland, 1994).

The microbiological quality of water used for washing or for brines is critical to production of a safe and stable product. Water with less than 100/mL total aerobic count when plates are incubated at 22°C and less than 10/mL total aerobic count when plates are incubated at 37°C has been deemed to be acceptable (Murphy, 1990).

It has been demonstrated that Listeria survive in a saturated brine solution held at 4°C for 132 days (Mitscherlich and Marth, 1984). Thus, brines used to salt butter must be free of Listeria. Water and brines used should also be free from pseudomonads. The most common form of spoilage in butter occurs with species of Pseudomonas (Jay, 1992; Mimer, 1995). Addition of salt to butter lowers the freezing point so that psychrotrophic microorganisms present may be able to grow at less than 0°C. Some psychrotrophic organisms multiply in salted butter stored as low as 21.2°F (−19.4°C) (Hammer and Babel, 1957). ·

The distribution of salt in the moisture phase of butter has less impact on growth of yeasts and molds on the surface of butter as compared to bacteria (Hammer and Babel, 1957). Humid conditions appear to have a greater impact on mold growth than does the material

on which they grow. Bacterial spoilage may occur in areas of low salt with large droplets of moisture in the past, many instances of spoilage in butter were traced to contaminated water supplies (Hammer and Babel, 1957). These instances typically involved organisms of the genus *Pseudomonas. Micrococcus* sp., many of which are lipolytic, have also been implicated in butter spoilage, especially of temperature-abused sweet cream butter (Varnam and Sutherland, 1994).

15.4.8 Packaging

In smaller operations, butter is loaded directly from the churn onto trolleys and wheeled onto packaging machines by plant personnel (Varnam and Sutherland, 1994). This type of packaging of butter exposes the product to air, workers, the plant environment, and temperatures that may promote spoilage. Control of the microbiological quality of air during packaging is therefore important. Practices that result in standing water or entrapped wet residues facilitate growth of environmental contaminants. Practices that aerosolize these contaminants afford a level of microbiological contamination to the air. Thus, practices that promote dry conditions in the plant are preferred. Numerous approaches can be taken to monitor microbiological air quality, which include sedimentation, impaction on solid surfaces, impingement in liquids, centrifugation, and filtration (Hickey *et al.*, 1992). Air quality is particularly important in the manufacture of whipped butter and in butter produced from continuous type churns that may incorporate up to 5% air into butter (if a vacuum deaerator is not used) (Varnam and Sutherland, 1994).

Personnel hygiene is also critical at this point of butter manufacture because contaminants from hands, mouth, nasal passages, and clothing may be transmitted to butter during packaging. Some continuous churns are arranged to discharge product directly into the receiving hopper of packaging machinery (Varnam and Sutherland, 1994). However, to ensure uninterrupted operation, it is more common to transfer butter to a silo via a butter pump. Some silos are sealed, but others are open. Sealed silos minimize the risk of further contamination from the plant environment but are of limited capacity (approximately 900 kg), whereas open silos have a capacity of up to 10 tons (Varnam and Sutherland, 1994). Butter must be pumped from these silos to the packaging equipment. Direct packaging into consumer-size containers is preferable over bulk packaging because such butter must be repackaged before sale. Such reworking can result in poor distribution of water droplets and thus increase the risk of spoilage (Mimer, 1995). A 1983 International Dairy Federation investigation of 79 butter factories indicated that 56% produced mainly 25-kg packages and 44% produced mainly consumer packages. However, two-thirds of these factories produced both types of packaged butter (Pointurier, 1986).

Cardboard boxes lined with vegetable parchment, aluminium foil, or a variety of plastic films are typically used for bulk packaging of butter (Varnam and Sutherland, 1994). Polyethylene is the preferred material based on its physical properties (low density, high impact, cost effectiveness, absence of copper, and nearly sterile condition) (Varnam and Sutherland, 1994). Parchment, which supports mold growth under humid conditions, is still frequently used (Varnam and Sutherland, 1994). Use of dry parchment or parchment treated 24 hours with 0.5% sorbic acid has been recommended (Varnam and Sutherland, 1994). Retail butter packs are typically wrapped in vegetable parchment or foil parchment laminate. Vegetable parchment is permeable to water. Thus, weight loss from water loss can occur during storage. Parchment allows penetration of ultraviolet light, which can

accelerate onset of oxidative rancidity (Varnam and Sutherland, 1994). Individual butter packs, used in restaurants and food service establishments, are made by filling preformed polyvinyl chloride pouches.

15.5 SPOILAGE AND UNDESIRABLE CHANGES IN BUTTER

Butter prepared from sweet unpasteurized cream contains the same microflora as the cream from which it was prepared. Also the bacterial changes which take place during storage are the same as those which occur in the milk or cream under the same conditions. Butter prepared from cream previously pasteurized at high temperatures, and then inoculated, generally contains only those organisms which were added to promote ripening. Some molds and yeasts may be present which result from air contamination. Since molds are generally aerobic, they grow chiefly on the surface of butter.

Undesirable changes which take place after butter has been manufactured are produced largely by the growth of organisms. Many of the organisms are present as a result of contamination of butter after its manufacture. Therefore, the same precautions followed in handling milk and cream also apply to butter. The extent of recontamination is roughly an indication of the care exercised in handling and storing butter.

Some butter defects and their causes are the following:

Surface taint—This is caused by *P. putrefaciens*, commonly found in raw milk and cream, butter, water, soil, and creamery equipment.

Rancidity—The first stage is believed to be a hydrolysis of the butterfat into glycerol and fatty acids. During this stage butter acquires a strong odour of butyric and caproic acids. A lipase capable of doing this is normally present in raw milk or is secreted by various contaminating organisms such as *P. fragi* and *P. fluorescens*. Since the enzyme is destroyed in the pasteurization process, the defect can be controlled by heat treatment.

Malty flavour—This is produced by a strain or variety of *Streptococcus lactis*. The organism has been isolated from raw milk. It is destroyed when milk or cream is pasteurized at 70 to 85°C for 30 min.

Skunk-like odour—This is produced by *P. mephitica*. The organism is presumably derived from the rinse water.

Fruity flavour—Fruity aromas may be imparted to dairy products by the growth of *P. fragi* in the milk or cream used in their manufacture.

Black discoloration—This is a defect produced by *P. nigrifaciens*. The organism produces a black to reddish-brown discoloration of butter. It is widely distributed in nature.

Surface discoloration—This is produced by molds of the genera *Cladosporium, Alternaria, Aspergillus, Mucor,* and *Rhizopus*.

Yeasty flavour—This is caused by the yeasts *Torula cremoris* and *T. sphaerica*.

Metallic flavour—This is caused by various organisms, including a strain of *S. lactis*.

Tallowiness in butter—A tallow-like odour is produced by oxidation. This may result from the action of ultraviolet rays of sunlight or from oxidases naturally present in milk. Certain mold enzymes are capable of producing a similar effect. Changes may be prevented by high-temperature pasteurization, which destroys the oxidases.

Control Measures for preventing Microbial spoilage in Butter

Following measures are very useful in controlling microbial spoilage of butter and related products.

(1)   Use of low count milk and cream.

(2)   Adequate pasteuization.

(3)   Efficient cleaning and sanitization of equipments.

(4)   Use of pasteurized water for washing.

(5)   Salting.

(6)   Uniform working.

(7)   Avoid reworking and blending.

(8)   Use of efficiently sanitized packaging materials.

(9)   Storage of butter at less than –12°C

(10) Air sanitation in butter making and packing rooms.

(11) Personnel hygiene.

15.6 QUALITY ASSURANCE

Any quality assurance program should incorporate maintenance and documentation of good manufacturing practices (GMPs) and hazard analysis critical control points (HACCP).

15.6.1 Hazard Analysis Critical Control Points

Obvious critical control points for butter manufacturers include pasteurization or repasteurization of cream received at the plant and control of the microflora in the manufacturing environment. Each plant must evaluate its individual process and develop its own risk assessment and HACCP plan (Smittle, 1992). An environmental sampling protocol should be aimed at monitoring this critical control point for L monocytogenes and Salmonella. Recalls of butter because of L. monocytogenes contamination were reported as recently as 1991 and 1992 (FDA Enforcement Report, 1991, 1992). Faust and Gabis (1988) have recommended areas of food plant environments that can be targeted for sampling. Presence of Salmonella and Listeria in the environment should result in corrective action with documentation of the success of that action. Irbe (1993) has recommended that manufacturers of whipped butter develop in-plant guidelines for aerobic plant-count and S. aureus at critical control points of manufacture. Finished products should be free of Salmonella, E. coli (Irbe, 1993), and Listeria.

Testing for these organisms can be done to validate success of the manufacturer's HACCP program. Manufacturers should also test for lipolytic and psychrotrophic spoilage organisms in the finished product and develop a three-class attribute sampling plan (Smittle, 1992) based on the principles of continuous quality improvement (Crosby, 1984). Sanitation of equipment used to manufacture product should be assessed on a daily basis by testing environmental swabs for their aerobic plate-count (incubated 3 days at 25°C). The lower temperature (25°C vs. 32°C or 35°C) allows for recovery of psychrotrophic bacteria as well as mesophiles.

15.6.2 Microbiological Standards

BIS Published microbiological criteria for butter include less than 50,000 aerobic plate-count per gram, less than 10 coliforms per gram, absence of *E. coli* type I in 1 gram, less than 10 yeasts and molds per gram, and absence of *S. aureus* in 1 gram (Anonymous, 1993). It has also been recommended that cream for butter making have an aerobic plate-count of less than 1000 per millilitre with less than 1 coliform, yeast, or mold per millilitre (Robinson, 1990).

15.7 MICROBIOLOGY OF RELATED PRODUCTS

15.7.1 Definitions

Margarines, like butter, contain approximately 81% fat, 15% moisture, 0.6% protein, 0.4% carbohydrate, and 2.5% ash (Irbe, 1993). In margarine, edible fats, oils, or mixtures of these whose origin is vegetable or rendered animal carcass fats are substituted for butterfat (Code of Federal Regulations, 1994). Both butter and margarine have fat contents in excess of 80%. This level is considered too high by many individuals concerned about their diets (Varnam and Sutherland, 1994). Consequently, a large number of spreads have been manufactured with lower fat contents. In many countries, there are no legal standards or definitions for these low-fat spreads. However, a working categorization has been made based on fat content (Varnam and Sutherland, 1994). **Full-fat spreads** are described as those with fat contents of 72% to 80%; **reduced-fat spreads** have 50% to 60% fat; **low-fat spreads** have 39% to 41% fat, and **very–low-fat spreads** have less than 30% fat. Vegetable fats, mixtures of vegetable fat and butterfat, and butterfat alone has been used to develop these spreads (Varnam and Sutherland, 1994). Another trend has been production of spreads in which fat has been replaced in part or completely by a variety of substances such as Neutrifat, Simplesse, and Stellar (Varnam and Sutherland, 1994). Olestra was recently (1996) approved by the Food and Drug Administration as a substitute for conventional fats and may appear in products in the future.

15.7.2 Dairy Spreads: Manufacture and Microbiological Considerations

Low-fat spreads are also w/o emulsions but contain a higher moisture content than butter. Consequently, these have increased likelihood of microbial growth unless preservatives are added. Preservative addition is allowed in some countries but not in others. Combining of ingredients occurs at 45°C in an emulsifying unit. This temperature may allow growth of thermoduric organisms (e.g., *Enterococcus faecium, Enterococcus faecalis)* and thermophiles. Higher fat dairy spreads are typically made using conventional continuous butter manufacturing methods. Margarine manufacturing techniques may be applied to high-fat vegetable oil spreads (Varnam and Sutherland, 1994).

Generally, these spreads are manufactured by adding moisture to the melted fat phase. The fat phase can be vegetable oil or a mixture of vegetable oil and butterfat alone (light butter) (Varnam and Sutherland, 1994). This is followed by addition of colour, flavour, and vitamins. Addition of 1% to 1.5% salt results in the same perception of "saltiness" as for salted butter (Milner, 1995). Pasteurization (typically 80°C to 85°C, 2-3 seconds) of the blend occurs after this step, given the potential for microbial growth and difficulty of cleaning

these emulsifying units (Varnam and Sutherland, 1994). However, pasteurization does not eliminate thermoduric or spore-forming microorganisms.

Crystallization of fat during working is critical to obtain the desired consistency of the finished product. Rapid supercooling (–10°C to –20°C) under high sheer conditions in a scraped surface heat exchanger initiates and maintains crystallization and disperses moisture within the fat matrix (Varnam and Sutherland, 1994). This cooling inhibits microbial growth. These products are often manufactured in an environment with filtered or sterile air. Control of cross-contamination during packaging is more critical than in butter manufacture because of the higher potential for microbial growth in spreads.

Microorganisms that cause spoilage in butter have also been implicated in margarine spoilage. However, vegetable fats are typically more resistant to lipolytic breakdown than butterfat (Varnam and Sutherland, 1994). *Yarrowia lipolytica. Bacillus polymyxa*, and *E. faecium* are spoilage organisms of concern in low-fat spreads (Varnam and Sutherland, 1994; Lanciotti et al., 1992). Lanciotti *et al.* (1992) showed that *L. monocytogenes* and *Yersinia enterocolitica* can grow in "light" butter at 4°C and 20°C. A class I recall of a 60% butter, 40% margarine product occurred in 1992 (FDA Enforcement Report, 1992). More detailed descriptions of margarines, spreads, and industrial milk fat products can be found in the report by Varnam and Sutherland (1994). An outline of margarine and spread manufacture is shown in Figure 15.2.
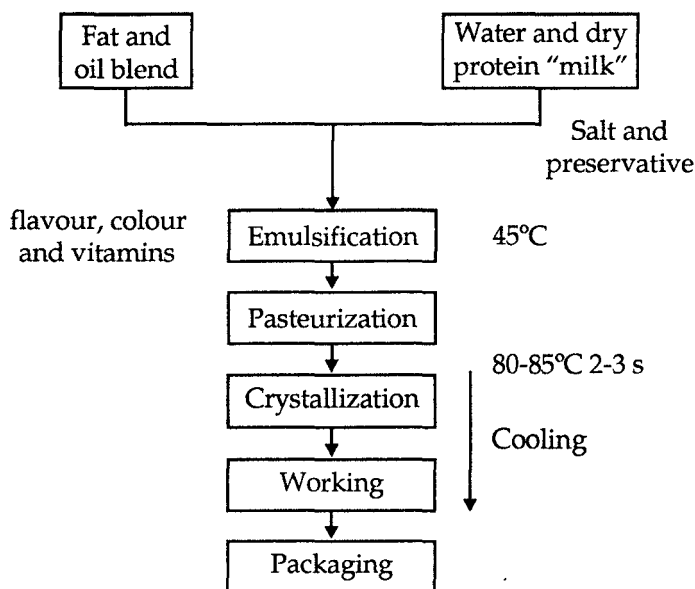
Fig. 15.2 : Production of dairy spreads (Adapted from Milner, 1995).

WHEY, CASEIN AND CASEINATES

16.1 INTRODUCTION

Whey, the fluid portion of milk drained from the curd in cheese or casein manufacture, may be sweet or acid. Of the 23 billion pounds of whey produced annually in the United States, about 25% is acid whey (pH 4.7) resulting from cottage cheese production. The remainder is sweet rennet whey (pH 6.2) from cheddar, Swiss and specialty cheeses. Casein whey, the U.S. production of which is small, contains the precipitating acid, either hydrochloric, sulphuric, or naturally-formed lactic. Billions of pounds of whey are produced in other countries notably in Europe, Australia, and New Zealand. In every whey producing area the problem of utilization vs. disposal into streams or sewers is being met by a vigorous search for new and profitable uses.

One hundred pounds of whole milk will produce about 10 lb of cheddar cheese and 90 lb of whey; 100 lb of skim milk will produce 16 lb of cottage cheese and 84 lb of whey or 2.8 lb of casein and 91 lb of whey.

16.2 COMPOSITION OF WHEY

Whey contains ½ the solids of the original milk; the fat and much of the protein are removed in cheese making. The composition of whey is shown in Table : 16.1. Whey is actually a 5% solution of lactose containing 2% of other milk components. It contains almost as much riboflavin as does milk. Acid whey contains more calcium and phosphate than sweet whey because of the solvent action of the acid used to precipitate the casein. The residual fat (0.3%) in sweet cheese whey is recovered by centrifugal separation. The protein is calculated from the nitrogen content of the whey or fractions separated from it. Whey, not treated to separate its proteins, contains β-lactoglobulin, α-lactalbumin, serum albumin, serum globulins, and other heat-denaturable proteins. Rennet treatment of the milk leaves a macropeptide in the whey which has a molecular weight of 8000; this macropeptide is split

from K-casein in the initial step of the clotting process. The proteins of whey may be separated by heat or heat plus acid and used in food preparation.

Table 16.1 : The composition of whey

	Sweet Cheese Whey	Cottage Cheese Whey	Casein Whey
Lactose	4.9	4.6	5.1
Heat-coagulable protein	0.5	0.5	0.6
Nonheat-coagulable nitrogenous matter	0.4	0.4	0.4
Ash	0.6	0.7	0.7
Fat	0.3	0.1	0.1
Lactic acid	0.2	0.6	—
Total Solids	7.0	7.0	7.0
Water	93.0	93.0	93.0

16.3 NUTRITIVE VALUE OF WHEY

The nutritive value of whey is high in some respects but low in others. The fat and most of the protein have been diverted to cheese. However, the biological value of the remaining whey proteins is higher than that of casein but their concentration is low. Excessive heat during processing, especially during the roller drying of whey, may lower its amino acid values by rendering them, especially the lysine, biologically unavailable. Most of the lactose and minerals remain in sweet whey, less in acid (fermented) whey. A lactose intolerance exists in some people who lack ability to hydrolyze the sugar; this gives rise to temporary abdominal discomfort. Since 73% of whey solids is lactose, the amount of whey that can be consumed by lactose -intolerant persons is limited. Whey solids can make a positive nutritional contribution to foods when used at a 3-10% solids level.

When whey is concentrated and dried there is approximately an 11-fold increase in solids and this concentration increase is reflected by an increase in most of the vitamins.

16.4 PROCESSING OF WHEY

Sweet whey must be processed within hours of its removal from the cheese curd to preserve its quality. Acid whey produced from casein or cottage cheese is more stable since lactic acid bacteria and many other organisms are inhibited in their growth at acidities below about pH 4.7.

The first step in processing is pasteurization which is usually followed by concentration and drying. Pasteurization is done at 82°-96°C. If denaturation of the whey protein is to be avoided temperatures below 74°C are used. The whey is concentrated under vacuum to 40-50% solids except in new reverse osmosis (RO) procedures. RO can be used to concentrate small quantities of whey (up to 100,000 lb per day) to 25% solids. This reduction in bulk facilities shipment to a central processing or drying plant where large

scale vacuum evaporating and drying equipment is available. There should be at least 100,000 and 750,000 lb whey per day available, respectively, to justify installation of a vacuum evaporator or spray dryer. Large whey drying operations will handle from one to several million pounds of whey daily.

Most of the whey that is processed is dried to preserve it for shipment, storage, and handling as a food or feed ingredient. For this purpose a non-hygroscopic product is desirable. Since 70% of dried whey is lactose, which normally dries as a hygroscopic glass, the lactose is generally crystallized in the concentrate (50% TS) and during the drying process. Whey dried from this concentrate yields a stable, crystalline sugar which will not absorb moisture as will the syrup form. The powder is packaged in multiwall bags of 50-100 lb capacity.

16.5 UTILIZATION OF WHEY

Sound factory and environmental practices demand that whey be salvaged for constructive purposes. Federal and state water quality standards have practically eliminated the practice of whey disposal into rivers and streams. Most municipalities are charging on a BOD basis for disposal into city sewers. Whey contains 6.3% organic solids. Its Biological Oxygen Demand (BOD) is the amount of dissolved oxygen taken up by the sample expressed in parts per million (ppm). The BOD measured over a 5-day period is expressed as BOD_5. The BOD_5 of 100 lb of cheddar cheese whey is 3.5 and the population equivalent is 21. Thus, 5 lb of whey is considered to cause pollution equal to that of the waste from an average individual. A cheese plant discarding 100,000 lb of whey per day would then require sewage disposal facilities the size of a town of 20,000 people. The importance of processing whey into useful feed or food products is clearly indicated.

Whey has long been fed to swine and other farm animals but part of the supply is now bringing better prices as a food ingredient. Lactic acid, alcohol, and vinegar were whey products for many years. Recently, however, whey has been largely displaced by less expensive fermentable materials. Whey is the raw material for lactose production and the amount of lactose manufactured is limited only by the uses that can be found for this unique carbohydrate. Small quantities of whey solids can be used in cheese spreads and in process cheese, and have been gradually displacing part of the non fat milk used in bakery goods, confections, and ice cream. It is especially adaptable to sweet baked hoods because it produces a soft cake-like texture. In ice cream, the addition of whey is permitted in an amount not to exceed 25% replacement of the non fat milk solids. When it is used to increase rather than replace milk solids, an improvement in body and flavour results. Ten to 15% of fudge or caramel solids can consist of whey solids to the improvement of both body and flavour of these candies. Nutritionally, whey could be an important ingredient in fruit-flavoured beverages and soft drinks.

Whey protein and lactose fractions are prepared for food manufacture by several fractionation or concentration processes. A high degree of purity often is not necessary when the whey product is to be used directly in food. Electrodialysis will take out part of the salts from which some of the lactose may have been already removed. Fractionation by ultra filtration (UF membranes, a form of reverse osmosis), is a newly developed procedure which has been made possible through the availability of improved long-lasting membranes. Typically, a fractionation membrane can remove 90% of the cottage cheese whey volume as

permeate containing 80% of the lactose and more than 90% of the ash. The remaining high protein fraction resembles skim milk in its proportions of lactose, protein, and salts. Thus, on a dry weight basis, if the original cottage cheese whey contained 68% lactose and 8% protein, a dry protein concentrate could be produced containing 33% protein and 52% lactose.

Gel permeation can be used to obtain high protein fractions by flowing the whey over a column packed with a suitable gel (Sephadex) which retains the smaller molecules of salts and lactose. The separation can be made more effective by first making an ultra filtration membrane separation. Such a concentrate may contain up to 80% of its solids as protein. Protein concentrations of 60-75% of solids can also be produced by diluting the first UF concentrate with water and refiltering it.

'Acido-whey' Beverage: The Whey Drink

Recently, National Dairy Research Institute (NDRI), Karnal (India) has prepared a beverage, namely acido-whey by converting whey through lactic acid fermentation. This beverage in palatable, refreshing and 'low-cost (priced at Rs. 1.00/200 ml in Sachet) for human-consumption. Lactic acid *(Lactobacillus acidophilus)* fermentation enhances nutritional properties and adds some therapeutic attributes to the beverage. However, cheese-whey is separated to remove fat and traces of curd particles, and heated to 95°C-100°C for 10-20 minutes. It is then cooled to 40°C, inoculated with an active culture of *Lactobacillus aciloptilus* and incubated at 30 ± 1°C for 20-24 hours. After incubation, fermented whey is clarified to remove precipitated cellular mass, sugar is then added to the product at the rate of 13%-14% in the form of 56% sugar syrup which has already been pasteurized. Desirable amount of flavour is added. The beverage is chilled and packaged in pouches.

Whey-Bran for Dairy Cattle

A whey-bran for dairy cattle has been prepared and used in Sweden. It is produced by drying a mixture of 33 kg. of rye-bran and 100 kg of fresh-whey. Whey in concentrated to 15-20% moisture before it is mixed with rye-bran. This mixture is then air dried.

Lactic Acid Manufacture

Cheese-whey can be used to produce lactic acid on large-scale. The microorganism used for whey-lactose fermentation is *Lactobacillus bulgaricus* the activity of which is enhanced by the associative action of mycoderm. Edible lactic acid prepared from whey is used in pickle manufacture and as an acidulant in bottled drinks, and 'sherbets'. Lactic acid is used to a comparatively minor extent in the farm of its salts, such as the lactates of calcium or sodium. Calcium lactate is used in pharmaceuticals, sodium lactate is used as a substitute for glycerol in textile industry.

The economic aspects of whey utilization largely govern its final disposition. Whey has many possible uses, but the cost of preparing it for special purposes must not cause it to lose its competitive position with alternative materials. It costs almost as much to dry and bag as it will bring as a feed ingredient (about 4¢ per lb). In unprocessed liquid form it can be fed to swine or recycled back to the cows, but again handling costs are scarcely recovered as available nutrients for the animal. Nevertheless, processing as feed at a break-even price is better than paying a BOD charge for waste disposal. Whey solids are most

valuable as human food, especially when high protein preparations are made from it. These too must be competitive with casein, vegetable protein, and egg white.

16.6 CASEIN

Casein in some form is doubtless one of man's oldest manufactured products. It is the major protein fraction of cow milk and along with other milk components has been one of man's most nutritious foods for thousands of years in the form of cheese. The housewife is most familiar with the product in its purest dairy food form, not as casein, but as "low fat" cottage cheese.

Casein comprises approximately 3% by weight of whole milk. In its commercial form, casein is isolated from the skim fraction of cow milk by any one of several methods. Although in appearance it is a homogenous material, casein is composed of at least four different fractions designated alpha, beta, gamma, and kappa and is one of the few proteins containing both sulphur and phosphorous. Casein in its natural form in milk helps to prov.de stability to the colloidal milk system in the form of calcium caseinate and probably calcium phosphocaseinate.

Recovery of casein by coagulation involves counteraction of one or more of the factors responsible for its stability.

In commercial practice this can be accomplished by the use of mineral acids such as muriatic (hydrochloric) or sulphuric acids, by lactic acid precipitation, and by the use of rennet. Desired properties of the finished product help determine the precipitating agent.

When acids are used precipitation occurs at pH 4.5-4.7 and this is referred to as the isoelectric point of casein.

Originally, all casein was produced by the vat or batch method whether produced from self-soured milk (lactic acid) or mineral acid use.

Later, however, continuous methods were devised. The lactic casein process can be described as a very large cottage cheese operation to which was added final pH adjustment to 4.6 with lactic or hydrochloric acid or sodium hydroxide as required, heating to 54°C to set the curd, separation from the whey, washing with warm water to remove salts and acid, separating again from the wash water, dewatering, drying, and sizing.

Rennet casein is produced by the enzyme action of the rennet on the milk casein and this product is subjected to the same separation, washing, drying, and sizing steps as used in the acid casein production.

Almost all casein produced until the late 1940's was for industrial applications such as glue and paper coating.

However, with improved processing equipment, sanitation, research, and imagination this changed. Now edible casein and caseinates have become important products. Today, an estimated 60-70% of the casein produced is for edible use in the form of casein or caseinates.

Casein is comparable in food value to the protein in meat and superior to vegetable proteins because of the better balance of its essential amino acids. A typical analysis for food grade casein is shown below:

Physical

Colour: light cream

Odour: essentially none

Water: 8-12%

Particle size: coarse granular, 30 mesh

fine granular, 80 mesh

Sediment and extraneous matter:

ADMI Standards

Chemical

Protein (N × 6.38): 83-84% (as is)

95-96% (dry basis)

Total ash (phosphorus fixed): 2.1%

Reducing substance (as lactose) 0.1-0.2%

Ether extractives: 1.5%

The use of edible casein, per se, is almost entirely nutritional. The fine mesh form (80 mesh) has been used successfully in fortifying bread and cereals.

Casein supplementation of wheat flour for bread at levels above 3% gives a coarse texture. A 1.1% supplementation gives the same protein and nutritional improvement to white bread as 3% supplementation with non fat dry milk and creates no problems with either batch or continuous mix breads as shown below:

	Bread Protein (%)	PER (protein efficiency ratio)	Improvement (%)
Water bread	7.8	0.7-0.8	
3% nonfat dry milk supplementation	8.3	1.0-1.1	40
1.1% casein or - sodium caseinate	8.3	1.0-1.1	40

16.7 CASEINATES

Because of its amphoteric properties casein reacts with acids or bases to form salts which dissociate in aqueous solutions.

Casein solublized with alkalies is known as caseinate. The two most widely used forms are sodium and calcium caseinate. These products are very useful because they are capable of providing both nutritional and functional roles in their use.

Water solutions of caseinates, 15-18% solids, with a pH of 6.6-7.2, are usually spray dried but they can also be roll dried.

The caseinates are used nutritionally to fortify and give texture to breakfast foods, breads, infant foods, and high protein diets. Functional as well as nutritional uses include usage in non-dairy coffee creamers, non-dairy whip toppings, confections, icings, firming the consistency of yogurt, and many other applications where smoothness and texture improvement are desired.

Sodium caseinate has been approved for use in imitation meat loaves, stews, and soups by the Meat Inspection Division of USDA. Certain states allow its use as a binder and emulsifier in comminuted meats, such as frankfurters, bolognas, and liverwurst. Caseinate, used at 1-3% level, prevents fat capping, shrinkage, and enhances the taste appeal of the product by promoting the retention of natural juices to provide a firm but succulent produce when cooked. In addition, it provides an additional quantity of protein of a quality equal to the meat itself.

In the years ahead it is believed that the superior nutritional and functional properties of casein and the caseinates will channel more and more of the world production into the edible market with industrial uses finding substitutes wherever possible.

The major casein producing countries are New Zealand, Australia, Canada, Argentina, France, Germany and Holland. Total estimated production is 200-500 million pounds per year.

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PROBIOTICS

17.1 INTRODUCTION

The Indian treatise "Sushruta Samhita" describes "dahi" as a food, promoting appetite and strength. The use of fermented milks in the human diet was also advocated by Metchnikoff, who attributed the longevity of Bulgarians to the ingestion of large quantities of Bulgarian milk prepared with the help of *Lactobacillus bulgaricus*. He further postulated that *Lactobacillus delbrueckii* subsp. *bulgaricus* possessed a therapeutic weapon to combat disease causing germs in human intestine. The scintillating observation paved the way for exploring the preservative potentials of lactic cultures and fermented products made from them in the alleviation of human and animal disorders.

Eli Metchnikoff (1908) theorized that *Lb. delbrueckii* subsp. *bulgaricus* could grow in the intestinal tract of humans and displace any putrefying bacteria that could grow there. Displacement of this group would reduce production of toxic compounds that adversely affects the human body, thus enabling humans to live longer. Research done since Metchnikoff's period has shown that *Lb. delbrueckii* subsp. *bulgaricus* neither survives nor establishes itself in the gastrointestinal tract. Other species of lactobacilli have been reported to provide some beneficial effects through growth and action in the gastrointestinal tract. This group of bacteria and others comprise the group referred to as probiotics. Cultures most often mentioned as probiotics for humans include *Lb. acidophilus, Lactobacillus casei,* and *Bifidobacterium* species. These all can survive and grow in the intestinal tract. Thus, through their growth or action in the intestinal tract, they have the potential to provide benefits.

Cultures normally used as starter cultures for some fermented milk products, such as *Lb. delbrueckii* subsp. *bulgaricus* and S. *thermophilus* used to manufacture yogurt, also may provide benefits, but not through the ability to survive and grow in the intestinal tract. Their function comes primarily from providing a source of enzymes necessary for improving the

digestion of nutrients in the gut. For example, β-galactosidase is needed for the initial breakdown of lactose in the small intestine (Gilliland and Kim, 1984).

Whereas there are reports that indicate that the nutritional value of milk can be improved by some fermentations (Hargrove and Alford, 1980), this chapter focuses on the potential health and nutritional benefits that rely on the growth or action of probiotic bacteria following their ingestion. Several benefits are possible from probiotic cultures, including control of intestinal infections, control of serum cholesterol levels, beneficial influences on the immune system, improvement of lactose utilization in persons who are classified as lactose maldigestors, and anticarcinogenic action. Research is continuing in each of these areas to provide definite scientific evidence that could permit specific health claims for dairy products containing one or more of this group of probiotic organisms. Several publications have focused on these in more detail. Foods containing such microorganisms may be promoted as functional foods in the future.

17.2 DEFINITION AND HEALTH BENEFITS

Probiotics are viable bacterial cell preparations or milk/food containing viable bacterial cultures or components of bacterial cells that have beneficial effects on the health of the consumer. Fermented milk has become a vehicle for probiotic bacteria.

Table : 17.1 lists the health benefits claimed for various probiotic lactic acid bacteria.

Table 17.1 : The health benefits claimed for various probiotic lactic acid bacteria

Benefits claimed	Probiotic strain
Prevention and treatment of gastro-intestinal bacterial diseases	Lb. casei Shirota
	Lb. rhamnosus GG
	Lb. acidophilus LC1
	Lb. salivarius WB1004
	Bifidobacterium breve
	Enterococcus faecium
Treatment of chronic urinary tract infection	Lb. acidophilus
	B. longum
	B. infantis
	E. faecalis
Stimulate immune responses	Lb. casei Shirota
	Lb. plantarum TCCI49I7
	Lb. fermentum Y1T0159
	Lb. acidophilus
	B. longum
	B. breve
	B. thermophilum
Treatment of constipation	Lb. rhamnosus GG
	B. breve

... contd.

Benefits claimed	Probiotic strain
Lowering liver cholesterol	*Lb. acidophilus* SBT2062
Treatment of hepatic encephalopathy	*Lb. acidophilus*
Prevention of diabetes	*Lb. casei* Shirota
Anti-hypertension	*Lb. casei* Shirota
Anti-cancer	*Lb. casei* Shirota
	Lb. rhamnosus GG
	B. infantis

17.3 POTENTIAL BENEFITS

17.3.1 Control of Growth of Undesirable Organisms in the Intestinal Tract

Lb. acidophilus, Lb. casei, and species of *Bifidobacterium* have been reported to inhibit growth of undesirable microorganisms that might be encountered in the gastrointestinal tract. Most of the older reports dealing with this type of control focused on a therapeutic approach, in that, cultured products made with these organisms were used to treat infections of various types (Gordon *et al.,* 1957; Winkelstein, 1955). Some of the studies involving these organisms were poorly done, and improper controls were used, so it is difficult to draw definite conclusions concerning the benefit of the probiotic organisms. The newer approach is to provide consumers with products containing the probiotic organisms as a preventive treatment in controlling intestinal infections. Studies using chickens as an animal model in which the birds were dosed with specific intestinal pathogens following consumption of cells of *Lb. acidophilus* have shown that the lactobacilli do exert control over *Salmonella* infections (Watkins *et al.,* 1982). Feeding the birds the lactobacilli before challenge with the pathogens, followed by continued consumption of *Lb. acidophilus* after the challenge dose, resulted in best control of the pathogens, suggesting that continuous consumption of the probiotic organism is desirable. The researchers conducting this study also showed that *L acidophilus* was effective in controlling *Escherichia coli* in the intestinal tract of chickens (Watkins *et al.,* 1982).

Just how the probiotic bacteria function in inhibiting the growth of intestinal pathogens in the intestinal tract is not clear. Many of the probiotic organisms produce substances that are inhibitory in vitro; however, it is difficult to confirm the activity of these compounds in vivo. The probiotic bacteria in question all produce large amounts of acids during their growth because they rely on fermentation to obtain energy for growth. However, the antagonistic action that they produce toward undesirable microorganisms apparently is not caused just by acids produced during their growth. Several of these organisms produce "antibiotic like" substances, some of which have been classified as bacteriocins, which may be involved in the antagonistic action toward the pathogens. Bacteriocins, according to the classical definition, are proteins produced by bacteria that are active against organisms closely related to the producer organism (Tagg *et al.,* 1976). This may limit the breadth of action of these inhibitory substances produced by probiotic bacteria toward a wide range of intestinal pathogens. They would not be expected to have any effect on gram-negative

intestinal pathogens. Furthermore, because of their sensitivity to proteolytic enzymes, bacteriocins may not survive to function in the intestines.

Competitive exclusion by the probiotic bacteria is another mechanism that has been suggested as being important in controlling intestinal infections (Watkins and Miller, 1983). Competitive exclusion involves the ability of the lactobacilli or bifidobacteria to occupy binding sites on the intestinal wall, thereby preventing attachment and growth of enteric pathogens.

Definitive scientific data showing the mechanism of action whereby these probiotic bacteria may exert inhibitory actions toward pathogens in the intestinal tract would make it easier to select the most effective strains of probiotic bacteria for use in dairy products to help control intestinal infections. Most likely, the antagonistic actions produced by the probiotic bacteria toward intestinal pathogens result from a combination of factors.

17.3.2 Improved Immune Response

Enhancement of the body's immune response by consuming cells of certain lactobacilli increases the resistance of the host to intestinal infections (Lessard and Brisson, 1987; Perdigon et al., 1990a; Satos et al., 1988). Of the lactobacilli, *Lb. casei* seems to be the primary one involved (Perdigon et al., l990b). As with other characteristics of the lactic acid bacteria, the relative ability of probiotic bacteria to cause such an effect probably varies tremendously among strains of individual species. Researchers in this area have suggested that this action involves activation of macrophages, which, in turn, destroy pathogenic organisms in the body. This enhancement of the immune system increases the host defense mechanisms and could be very important in the control of food-borne illnesses. This may be a key explanation as to how certain probiotic microorganisms used as dietary adjuncts can exert control over intestinal infections.

17.3.3 Improved Lactose Digestion

People who lack the ability to adequately digest lactose are classified as lactose maldigestors. (In the past, terms such as "lactose intolerance" or "lactose mal-absorption" have been used to describe these individuals.) The problem results from inadequate levels of β-galactosidase in the small intestine to adequately hydrolyze ingested lactose. Once a lactose maldigestor ingests sufficient lactose, it passes into the large intestine where it undergoes an uncontrolled fermentation that results in symptoms of cramps, flatulence, and diarrhea. These symptoms often follow consumption of milk by such individuals. Because lactose maldigestion is a result of inadequate levels of an enzyme to hydrolyze lactose in the small intestine, the possibility exists for providing such an enzyme in the diet. Inclusion of a purified enzyme such as β-galactosidase in a diet would be rather expensive, and survival of the enzyme during passage through the stomach likely would be minimal. Research has shown that the presence of viable starter cultures in yogurt can be beneficial to lactose maldigestors (Gilliland and Kim, 1984; Kolars et al., 1984). This beneficial action has been shown to result from the presence of β-galactosidase in bacterial cells. Apparently, being inside the bacterial cells protects the enzyme during passage through the stomach so that it is present and active when the yogurt reaches the small intestine. Once the yogurt culture reaches the small intestines, it interacts with bile, which increases the permeability of cells of these bacteria and enables the substrate to enter and be hydrolyzed (Noh and Gilliland, 1992). The enzyme remains inside the cell upon exposure to bile rather than leaking out

into the surrounding medium. As discussed, the starter cultures used for yogurt manufacture *(Lb. delbrueckii* subsp. *bulgaricus,* and S. *thermophilus)* do not have bile resistance and thus are not expected to survive and grow in the intestinal tract. Despite this limitation, consumption of these bacteria does provide a means of transferring β-galactosidase into the small intestines where it can improve lactose utilization in lactose maldigestors.

Non-fermented milk containing cells of *Lb. acidophilus* also can be beneficial for lactose maldigestors (Kim and Gilliland, 1983). This organism, unlike the yogurt starter cultures, can survive and grow in the intestinal tract. However, a similar mechanism in improving lactose utilization in lactose maldigestors to that observed of yogurt bacteria is probably involved. β-galactosidase activity of *Lb. acidophilus* is greatly increased in the presence of bile (Noh and Gilliland, 1993). As with the yogurt culture, cells of *Lb. acidophilus* do not lyse in the presence of bile but their permeability is increased, permitting lactose to enter the cells and be hydrolyzed. Because *Lb. acidophilus* can survive and grow in the intestinal tract, it is reasonable to expect, however, that additional β-galactosidase may be formed after ingestion of milk containing this organism.

There has been some controversy over the benefit of acidophilus milk in being effective in improving lactose utilization in lactose maldigestors; however, if the cells contain sufficient levels of β-galactosidase before ingestion, it is reasonable to assume they would provide such a benefit. Some studies that have suggested that milk containing *Lb. acidophilus* is ineffective (Payne *et al.*, 1981; Saviano *et al.*, 1984) in improving lactose digestion might be questioned because no evidence was provided concerning the cultures used or the procedure by which they were produced. It is possible, in those studies, that insufficient β-galactosidase was present in the milk containing cells of *Lb. acidophilus* at the time of consumption. One of the studies (Saviano *et al.*, 1984) indicated that no β-galactosidase activity was detected in milk containing *Lb. acidophilus.*

Based on the proposed mechanism for improving lactose digestion by yogurt cultures, it seems reasonable that any product containing bacterial cells having adequate intracellular β-galactosidase activity could provide a benefit such as improving lactose utilization. Because this enzyme usually is inducible in most microorganizations, it is important that the organisms be grown in the medium containing lactose before ingestion. This becomes particularly important when cells of probiotic bacteria grown in some medium other than milk are added to non-fermented milk. The level of β-galactosidase activity also varies among strains of *Lb. acidophilus* as well as among commercial yogurt cultures. Therefore, it is important to consider the level of β-galactosidase activity in probiotic or starter cultures to be used for improving lactose digestion in lactose maldigestors. It is also important for the activity to remain high during transportation and storage of such products so that the consumer receives the product containing enough of the enzyme to provide a benefit.

17.3.4 Anticarcinogenic Actions

Anticarcinogenic or antimutagenic activities have been reported for several cultures used to manufacture various fermented milk products (Goldin and Gorbach, 1984; Oda *et al.*, 1983; Reddy *et al.*, 1983; Shahani *et al.*, 1983). Some of these have involved products containing probiotic bacteria expected to survive and grow in the intestinal tract, whereas others have involved the use of fermented products manufactured when organisms not normally expected to survive and grow in the intestinal tract. For instance, consumption of

yugurt by mice inhibited development of certain tumours (Reddy *et al.*, 1983). In other studies involving human subjects, a culture of lactobacilli exhibited potential in controlling cancer of the colon (Goldin and Gorbach, 1984). The lactobacillus used in this study was later identified as *Lb. casei*.

Lb. acidophilus, *Lb. casei*, and *Lb. delbrueckii* subsp. *bulgaricus* are the species most often mentioned as having potential to produce or provide anticarcinogenic actions. For *Lb. delbrueckii* subsp. *bulgaricus*, the anticarcinogenic action apparently is associated with substances formed by the organism during fermentation of the yogurt as opposed to being produced in the body following consumption of the yogurt. However, for *Lb. acidophilus* and *Lb. casei*, growth or action in the gastrointestinal tract seems to be important. Part of the benefit may involve direct effects on inhibiting tumour formation; however, the main effect may result indirectly through inhibition of the growth of undesirable bacteria that may form carcinogens in the large intestine (Goldin and Gorbach, 1984). Thus, this may represent another benefit from being able to control growth of undesirable organisms in the gastrointestinal tract.

17.3.5 Control of Serum Cholesterol

In the 1970s, two studies revealing that organisms such as *Lb. acidophilus* can have potential in reducing serum cholesterol levels in humans were published. One of these involved milk fermented with what was described as a "wild" strain of *Lactobacillus* and then fed to a group of men on a high-cholesterol diet (Mann and Spoerry, 1974). The study was designed to study the influence of a surfactant (Tween 20) on serum cholesterol levels. The researchers theorized that the surfactant would increase absorption of cholesterol from the intestine and thus increase serum cholesterol levels. Conversely, the serum cholesterol level in both groups of men, that is, those receiving the surfactant and those who did not, decreased. This was one of the first studies that suggested consumption of a fermented dairy product could reduce serum cholesterol levels in humans. However, neither the organism involved in the fermentation nor the mechanism was identified. In another study, cells of *Lb. acidophilus* added to infant formula reduced serum cholesterol in infants receiving the formula (Harrison and Peat, 1975), whereas infants receiving the formula without cells of *Lb. acidophilus* exhibited increased serum cholesterol levels. The researchers concluded that *Lb. acidophilus*, through its growth in the intestine, in some way influenced the serum cholesterol level, although no mechanism was suggested.

Several animal feeding studies have shown that consumption of milk containing cells of *Lb. acidophilus* by animals resulted in lower serum cholesterol levels than in animals which did not receive milk containing the lactobacilli (Danielson *et al.*, 1989; Gilliland *et al.*, 1985; Grunewald, 1982). There is variation among strains of this organism in their ability to exert control over serum cholesterol levels (Gilliland, 1989). Some strains of Lb. *acidophilus* can actively assimilate or take up cholesterol during growth in laboratory media. This occurs when the organisms are grown anaerobically in the presence of bile, conditions that occur in the intestinal tract. Pigs on a high-cholesterol diet fed a strain of *Lb. acidophilus* that actively assimilated cholesterol during growth in laboratory media had significantly lower serum cholesterol levels than did pigs receiving a strain of *Lb. acidophilus* that did not actively assimilate cholesterol in laboratory media. This suggests that the ability to assimilate cholesterol in laboratory media provides an indication of the potential of this organism to exert some control over serum cholesterol levels.

Another activity of *Lb. acidophilus* that may be important is its ability to deconjugate bile acids. This provides yet another mechanism whereby *Lb. acidophilus* might exert control on serum cholesterol levels. The deconjugation of bile acids by lactobacilli can occur in the small intestines. Free bile acids are less well absorbed from the small intestines than are conjugated ones and thus more are excreted through feces (Chickai *et al.*, 1987). Excretion of bile acids through feces represents one of the major mechanisms whereby the body eliminates cholesterol. This is because cholesterol is a precursor for synthesis for bile acids and any bile acids that are excreted from the body are replaced by synthesis of new ones. Thus, there is a potential for reducing cholesterol pool in the body. Furthermore, free bile acids do not support the absorption of cholesterol from the intestinal tract as well as the conjugated ones (Eyssen, 1973), thus deconjugation of bile acids in the intestinal tract may reduce the efficiency of absorption of cholesterol from the intestinal tract.

Research into the potential of *Lb. acidophilus* to exert hypocholesterolemic effects, in humans has indicated tremendous variation among strains of *Lb. acidophilus* isolated from the human intestinal tract in their ability to assimilate cholesterol (Buck and Gilliland, 1994). Evaluation of strains of *Lb. acidophilus* currently used commercially in cultured or culture-containing dairy products in the United States has revealed that none of them are particularly active with regard to actively assimilating cholesterol in laboratory media (Gilliland and Walker, 1990). On the other hand, new strains that are very active in this regard have been isolated from the human intestinal tract and thus they may provide greater potential for use as dietary adjuncts to assist in controlling serum cholesterol levels (Buck and Gilliland, 1994). From 122 isolates of *Lb. acidophilus* obtained from human intestinal sources, several were identified as having great potential for exerting control over serum cholesterol levels because they were very active in assimilating cholesterol during growth in a laboratory medium. They were far more active in this regard than were the currently commercially available strains of *Lb. acidophilus*. One of these strains has been used by a company in Holland to produce a fermented yogurt named *Fysiq*, which has been promoted as being useful in helping maintain a healthy cholesterol level. This strain has been used in a human feeding trial of hypercholesterolemic individuals and effectively caused a significant reduction in serum cholesterol levels (Anderson *et al.*, 1997).

There may be possibly other probiotic organisms that can exert benefits in controlling serum cholesterol levels. Some of these include *Lb. casei* as well as *Bifidobacterium* species. Cultures in both of these species can remove cholesterol from laboratory media during anaerobic growth in the presence of bile. They also are effective in deconjugating bile acids. Currently, there is great interest throughout the world in the potential of these bacteria exerting some control over serum cholesterol levels in hypercholesterolemic individuals.

17.4 CHARACTERISTICS NEEDED FOR PROBIOTIC CULTURES

There is potential for probiotic cultures to provide health and nutritional benefits for consumers. However, data are insufficient in most instances to permit specific health claims in the United States for dairy products containing them. The improvement of lactose utilization in lactose maldigestors is the possible exception. Before specific health claims can be made for most of these products, it is necessary for clinical trials to establish that the benefit indeed occurs. Such trials should be conducted using only probiotic bacteria that have been selected for a specific activity. In other words, they should be selected in some manner to produce the desired health or nutritional benefit (Gilliland and Walker, 1990). It

is unreasonable to expect one strain of any of the species involved to provide all of the potential health or nutritional benefits. In the past, most of the knowledge gained concerning variations among strains of species of starter culture bacteria has focused on the ability of these organisms to produce desired organoleptic properties in cultured products and to do so as rapidly as possible. Very little, if any, attention has focused on the potential health or nutritional benefits possible from these cultures. Most strains of the probiotic bacteria commercially available have not been selected for any specific activity except to, perhaps, have their identity confirmed as the indicated organism. To be successful as probiotic cultures, they must be selected for their ability to provide the targeted benefit for the consumer.

If cultured or culture-containing dairy products are to be useful as functional foods in providing health or nutritional benefits for consumers, it is necessary to alter the basis used for selecting starter culture bacteria. The cultures not only must be selected for their ability to produce the desired organoleptic properties in the cultured product but factors related to their potential health or nutritional benefits must also be considered (Gilliland and Walker, 1990). The primary factor to be considered in the selection is that the culture must be able to produce the desired benefit. Furthermore, the culture should retain that ability during production, manufacturing, distribution, and storage of the product before reaching the consumer. If the desirable action requires that the organism grow in the intestinal tract, then the characteristics that enable the organism to grow well under these conditions must be considered. To help ensure the ability of the organism to establish or grow in the intestines, it is important to consider the bile tolerance of the strain selected. The probiotic bacteria under consideration tend to be host specific; therefore, it is necessary to consider the source from which the organism came. In other words, it is desirable to select the strain that is compatible with the host (i.e., humans) for which the product is intended.

In some instances, a product such as yogurt, which is made with the traditional yogurt culture, *Lb. delbrueckii* subsp. *bulgaricus* and S. *thermophilus,* also is supplemented with cells of *Lb. acidophilus* and/or *Bifidobacterium* species. If such a procedure is implemented to provide the consumer with the beneficial organisms, then care must be exercised to ensure that adequate numbers of probiotic organisms are present. Some of the probiotic cultures with the potential for providing health and nutritional benefits may not grow as well in milk during the manufacture of fermented milk products as some of those that have been traditionally used for producing such products. Thus, research may be necessary to determine ways to improve the growth of the probiotic organisms in the milk being fermented so that the consumer is provided with adequate numbers of these potentially beneficial bacteria.

For any of the potential health or nutritional benefits that might be derived from probiotic cultures, it is necessary to develop proper testing of the cultures and the products containing them to ensure that the consumer receives the product that is most likely to provide the intended benefit. If such products as functional foods are to be effective, it is necessary to use the properly selected probiotic cultures. This may result in the requirement of several types of yogurt or other fermented milk products, each containing a different selected strain or strains of the probiotic culture to provide the specific desired health or nutritional benefits.

□□□

18

PHYSICO-CHEMICAL TESTING OF MILK AND DAIRY PRODUCTS

18.1 INTRODUCTION

Physico-chemical testsing in the dairy plant is critical to ensure that raw milk, other ingredients, and finished dairy products are of high quality. Such testing also serves to verify the adequacy of Hazard Analysis Critical Control Point (HACCP) procedures. This chapter briefly explains various physico-chemical tests that might be done on incoming raw milk as well as various types of dairy products, such as butter, ice-cream, and cheese. Tests for detecting adulterants in milk and dairy products are also briefly mentioned.

18.2 ANALYSIS OF MILK

18.2.1 Fat (Volumetric Gerber's Method)

Milk is added to sulphuric acid to dissolve casein. The mixture is centrifuged and separated fat is measured on the scale directly. Amyl alcohol is added to reduce charring of organic matter (hence instead of concentrated H_2S0_4, 90% acid used) and to facilitate separation. To two milk butyrometres add the following reagents: 10 ml of Gerber's sulphuric acid, 11.04 ml of well mixed milk sample, 1 ml of amyl alcohol. Insert, the stopper and shake till no white particles are seen. Place the butyrometre (Fig 18.1a) in water at 65 ± 2°C. Centrifuge for 4 to 5 minutes at 1100 rpm and again keep in a water bath for 3-10 minutes. Remove the butyrometre and read fat percent to nearest 0.05.
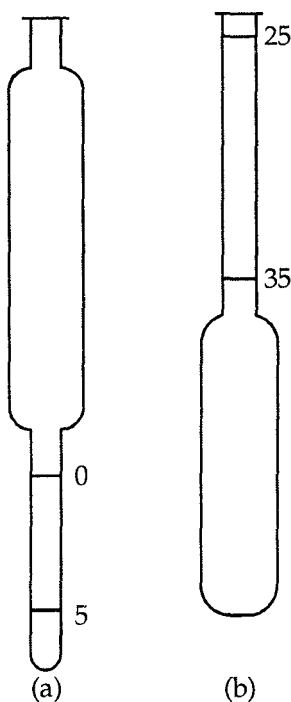
Fig. 18.1 : (a) Gerber butyrometer (b) Density lactometer.

18.2.2 Total Solids in Milk

The moisture in milk is evaporated in an oven and the residue is reported as total solids.

Weigh accurately 10 ml of mixed milk in a round, flat porcelain dish. Keep it on a boiling water bath for 30 minutes. Transfer it to an air-oven at 100°C, and dry for 2 hours. Cool and weigh. Heat the dish as before for 1 hour, cool, and reweigh to a constant weight. Calculate percentage of total solids. The total solids can also be calculated using the modified Richmond equation given below:

Total solids = [0.25 × lactometre reading at 20°C + (1.22 × percent fat) +0.72]

The value lies between 11.4 - 14.5%.

18.2.3 Titratable Acidity

The titratable natural acidity of milk is determined by titrating with 0.1 N sodium hydroxide, using phenolphthalein as an indicator.

Take 10 ml of milk in a porcelain dish. Add 1 ml of phenolphthalein. Titrate the solution against 0.1N NaOH. Calculate acidity as percentage lactic acid. 1 ml 0.1N alkali = 0.009g lactic acid. Fresh milk gives titre of 1.4 - 1.8ml.

18.2.4 Specific Gravity

Cow's milk has a specific gravity value of 1.032. The milk constituents influence this value. Milk solids non-fat (1.5) tend to raise this value and fat particles (0.9) lower it.

The value decreases on addition of water. Using this, the volume of milk can be converted into weight. The value is further useful in calculating total solids. For measuring, it special hydrometer called a lactometer is used. It has a scale graduated into 25 equal parts, starting from 15 upto 40. These divisions represent specific gravities from 1.015 to 1.040. For converting the lactometer reading to the specific gravity value 1.0 is prefixed to the reading.

Dip a clean lactometer into the milk sample and note down the lactometer reading. Prefix 1.0 to the reading and read as specific gravity.

18.2.5 Ash Figure

Milk ash is a fairly constant figure. The average value is very close to 0.7%.

Weigh accurately 20g of milk in a platinum or porcelain dish. Add 6 ml of concentrated HNO_3 to it. Place the dish on a steam bath and evaporate to dryness. Transfer to a muffle furnace at 600°C and heat to white ash. Cool and weigh the residue and report as percentage ash.

18.2.6 Chloride Value

Higher chloride amounts are indicative of the diseased state of an animal (mastitis), a poor quality of source, or reconstituted and tampered sample. Weigh accurately 10 ml of milk in a conical flask. Add 40 ml of distilled water, 10 drops of 10% K_2CrO_4 solution indicator and titrate with 0.1N $AgNO_3$ solution. 1 ml 0.1N $AgNO_3$ = 3.55 mg chlorine.

18.2.7 Estimation of Lactose

Lane Eynon's volumetric method—The method is based upon the reducing property of lactose in milk. Lactose reduces Fehling's solution. The titration is carried out by Lane Eynon's method.

Weigh accurately 10 g of milk and transfer it to a 250 ml volumetric flask with 50 ml water. Add 5 ml each of $K_3Fe(CN)_6$ and zinc acetate clearing agent, to precipitate casein and fat which would otherwise interfere. Adjust the volume to 250 ml with distilled water and filter. Titrate 10 ml of Fehling's solution against this solution by Lane Eynon's method.

1 ml of Fehiing's solution = 0.00645 g of anhydrous lactose.

Iodometric method—The method is based upon the ability of I_2 to oxidize aldose sugars e.g. lactose and glucose to corresponding carboxylic acid. The oxidation occurs in presence of sodium hydroxide. From the amount of iodine required, for oxidation, the sugar content can be estimated. To the clarified milk sample excess of iodine solution is added, and the solution is made alkaline with NaOH. The reaction is allowed to go to completion for 20 minutes. The remaining alkali is neutralized and excess iodine is back titrated with $Na_2S_2O_3$.

Weigh 10 g of milk and transfer to a 100 ml volumetric flask with 50 ml of distilled water. Add 10 ml Meyer's reagent and 2 ml of dilute H_2SO_4 (fat, protein clarifying agent). Adjust the volume to 100 ml with distilled water and filter the solution. Neutralize 25 ml filtrate with 1.5% NaOH. Add 20 ml 0.1N I_2 solution and then 30 ml of 0.1N NaOH solution. Keep in the dark for 20 minutes, Add 4 ml dilute H_2SO_4. Titrate the excess of I_2 with 0.1N $Na_2S_2O_3$ solution using starch as an indicator. 1 ml of 0.1n I_2 = 0.01705 g lactose.

18.2.8 Heat Stability Test

These tests are designed to determine whether the given milk sample will withstand sterilization and pasteurization processes or not, thus its suitability, for heat treatment.

Alcohol coagulation test—It is useful in condensing and sterilization process. Alcohol, at a high concentration precipitates casein from milk. This is affected by pH, salt balance, protein breakdown etc., and is taken as an index of stability. For fresh milk it is greater than 80%.

In a test tube take 5 ml of milk. To it add 5 ml of 70% alcohol rapidly. Check the solution for any coagulation; which indicates non-suitability of milk.

Phosphatase coagulation test—For determining the stability of milk, under sterilization conditions, phosphatase coagulation test is used.

Take 10 ml of milk in a test tube. Add 1 ml of phosphate solution (KH_2PO_4 68.1 g / litre of distilled water). Mix well and immerse in a boiling water bath for 5 minutes. If any coagulation occurs it indicates poor heat stability.

18.2.9 Heat Adequacy Test

Phosphatase test—It is used to assess the adequacy of the pasteurization process. Phosphatase enzyme, present in raw milk, gets inactivated during heating. These enzymes are more difficult to destroy.

Take 5 ml of buffer substrate solution in a test tube. Add 10 ml of milk to it. Stopper the tube and adjust the temperature to 37°C. Mix the contents well. Incubate the solution at 37.5°C for 2 hours. Run a blank using 10 ml of boiled milk sample. A dark yellow colour indicates the presence of enzymes, and thus insufficient heating.

Turbidity test—The test is designed to determine the extent of sterilization process. The milk is shaken with $(NH_4)_2SO_4$, filtered and filtrate is heated in boiling water. During sterilization, albumin gets denatured. If any denatured albumin remains present due to inadequate heating, it separates out with casein, resulting in turbid solution.

Weigh 4.1 g of ammonium sulphate in a 50 ml conical flask. Add 20.5 ml of milk sample, shake for 1 minute and keep for 5 minutes. Filter the solution using Whatman No 12, filter paper. Collect 5 ml of clear filtrate, and keep it in a beaker of boiling water for 5 minutes. Cool the solution and view for turbidity. A turbid solution indicates non-denatured albumin.

18.2.10 Proteins (Formol Titration Method)

Amino acids constituting the proteins are too weak to be titrated directly with alkali. However, when formalin is added, the NH_2 group is replaced by a methylene imino group, (N-CH_2) and the COOH group becomes available for titration.

$$R.COCH. NH_2 + HCHO \longrightarrow H_2O + RCOCH.N = CH_2$$
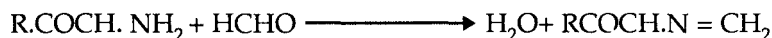
Take 10 ml of milk in a conical flask. Add 0.5 ml of phenolphthalein indicator and 0.4 ml of neutral saturated K-oxalate solution (to overcome calcium interference). Neutralize the solution with 0.1N NaOH ($V_B$ml). Add 2 ml of formalin solution (neutralized) mix well and keep for 5 minutes. Titrate the acidity produced with 0.1N NaOH using neutral phenolphthalein indicator ($V_S$ml), calculate percentage protein as = 1.7 (V_B-V_S).

18.3 TEST FOR ADDED WATER

The method is based upon measuring the change in the refractive index of milk when water is added. Milk serum (milk without fat and protein) due to its more uniform composition is preferred for measurement. Serum is obtained by addition of $CaCl_2$, acetic acid or $CuSO_4$, when proteins get precipitated. Use of $CuSO_4$ is preferred since the action is rapid and gives constant results. 10% added water can be detected by this method.

Prepare a 7.25% $Cu50_4$ solution in water. Dilute it further to give an immersion refractometre reading of 36 on the scale or a specific gravity value of 1.0443. Mix the milk and $CuSO_4$ solution in the ratio of 4:1 *(V/V)* and filter the solution. Determine the RI of the filtrate using an immersion refractometre. Minimum values for copper serum of normal milk are refractometre reading, 36 specific gravity 1.0245 and total solids 5.28%. A refrectometre value of less than 36 is suggestive of added water.

Added water can be quantitatively determined by the cryoscopic method which measures the change in freezing point due to added water. Milk freezes at an average temperature of 0.55°C (Range 0.5 to 0.61°C) and the freezing point is almost constant. Addition of 1% water to milk decreases the freezing point by 0.0055°C.

18.4 PRESERVATIVES IN MILK

Milk preservatives are or two kinds, those meant for addition in the samples for chemical analysis and those added for prolonging the shelf-life of stored milk. In case of analytical samples, colouring agents are added to make it distinctly different. In such samples the employed preservatives are HCHO, $CHCl_3$, ether, fluorides, K_2CrO_4, $HgCl_2$ and H_2O_2. The use of these two categories of additives is permissible under law.

Use of preservatives, in samples meant for human consumption is non-permissible under law. The commonly employed ones are $NaHCO_3$, H_2O_2 and occasionally formalin. They help in selling old milk as fresh milk. The developed defects like acidity, can be concealed by their use.

(1) Hydrogen Peroxide—Its use as preservative is common. To the milk sample add an equal volume of Concentrated HCl and mix. Add a drop of dilute formalin solution and warm the mixture to 60°C. Dip starch iodide paper in the solution. If the paper turns blue or violet it indicates added H_2O_2.

(2) Boric Acid (Borax)—Take the milk sample in a test tube and acidify with dilute HCl. Dip turmeric paper in the sample. Allow the paper to dry. If boric acid is present, the paper turns red.

(3) Formaldehyde—Take 10 ml milk in a test tube. Add 5 ml concentrated H_2SO_4 along the sides of tube to form a layer. A violet/blue colour formed at the junction indicates added HCHO.

(4) Benzoic Acid—Curdle the milk sample by adding Dilute HCl (20:1 ratio). Filter and extract filtrate with 50 ml ether. Wash with water and evaporate the solvent on water bath. Shining flakes of benzoic acid, with a typical odour, may be observed on heating. Dissolve residue in hot water. Make it alkaline with ammonia. Remove excess ammonia and add a little hot water to dissolve. Add neutral $FeCl_3$ solution. A salmon coloured precipitate indicates added benzoic acid.

(5) **Hypochlorites**—Hypochlorite residues may get introduced due to the use of Cl_2 sterilizers. Cool 3 ml milk to 3°C. Add equal volume of $SnCl_2$ solution at 3°C (0.025% in 73.5% H_2SO_4). Shake well and keep at 3°C. Centrifuge the solution for 3 minutes. A yellow green colour indicates hypochlorites.

(6) **Salicylic Acid**—Curdle the milk with dilute HCl (20:1) Filter and extract with 50 ml ether. Wash ether twice with water. Evaporate ether layer on a water bath and add one drop of $FeCl_3$ solution. A violet colour formed indicates added salicylic acid.

(7) **Lactose : Polarimetric Method**—Weigh accurately 65.8 g of milk in two standard flasks. Add to each 20 ml of acidic $HgNO_3$ ($HgNO_3$: HNO_3 : H_2O 1:2:5) and 30 ml of mercuric iodide solution. To one flask add 5% phosphotungstic acid solution to make the volume upto 100 ml. To the other add 15 ml of phosphotungstic acid solution and dilute to 200 ml. Shake well and keep for 15 minutes. Filter and note the rotation. Subtract from the other reading and multiply by 2 subtract this from the 1st reading (solution with 100 ml volume). Divide the reading by 2 and report as percent lactose.

(8) **Carbonates/Bicarbonates**—Being alkaline they help in neutralizing the acidity and the bacterial activity. Evaporate to dryness 5 ml of milk in a dish. Ignite it to white ash. Dissolve the ash in water and titrate with standard acid. If greater than 0.3 ml of acid is required, it indicates added alkali.

18.5 ADULTERANTS IN MILK AND TESTS TO DETECT THEM

The main adulterants of milk are water, thickening agents, skimming and water addition, colouring matter in buffalo milk, milk powder, preservatives and neutralizing agents, cow's milk sold as buffalo milk. Addition of water decreases the specific gravity, fat, N, SNF and lactose values and depresses the freezing point.

Partial skimming has the same effect. Thickening agents increase the SNF value, and the assay value of N and sugar may be less. Skimming (fat removal) lowers fats%, increases density reading and raises the ratio SNF:fat.

Added skimmed, milk decreases fat percentage, increases density, increases percentage of SNF, and ratio of SNF: fat.

(1) **Estimation of Fat**—The fat content of the suspected sample is determined and the deficiency is calculated. Values less than 4.5 for cow's milk and 6.5% for buffalo milk indicate adulteration.

(2) **Vieth's Ratio**—For normal milk the Vieth's ratio (Lactose : Ash : Protein) is constant (6 : 5 : 1 for buffalo milk). If added water is present, the SNF value decreases but the ratio remains unaltered.

(3) **Refractive Index Value (RI)**—RI value is a function of the concentration of dissolved substances in milk. With added water, the RI value decreases since concentration decreases.

For genuine milk serum, RI value is 1.4 and for water it is 1.33. With added water, the RI value approaches 1.33. For genuine milk serum, the refractometre reading value is between 38.5 to 40.5. With added water it decreases to below *38.5*.

SNF Determination—For normal buffalo milk the SNF value is 9.17. With added water this figure gets reduced.

percentage water added is calculated as equal to $100 - \dfrac{SNF}{9.5} \times 100$

Where SNF = Solids non-fat.

(4) Protein, Fat Ratio Measurement—The normal value of this ratio is less than one (Fat> Protein). A value exceeding 0.9 is indicative of partial skimming or addition of separated milk.

(5) Freezing Point Method—This method is useful in determining added water. Added water lowers the concentration of lactose and minerals raising the freezing point. Buffalo milk sold as cow milk can be detected by this method.

percentage water added $= 100\dfrac{(T-t)}{T}$

T = Freezing point (°C) of a genuine sample

t = Freezing point (°C) of given sample

(6) Organoleptic Test—Quality of the milk can be assessed by its smell, colour and taste. Smell the milk immediately on its arrival. A foul or abnormal smell may be indicative of poor quality of milk. Observe the colour of sample. Abnormal colour may indicate suspicious quality of sample. Also observe the sample for following taints:

1. Due to developed acidity.
2. Due to feed or due to exposure to stable atmosphere.
3. Due to extraneous matter.
4. Oxidized flavour due to light or metallic contamination.

(7) pH—The pH value gives a measure of the true acidity of the milk. The normal value ranges between 6.6-6.8. Developed acidity decreases the value. Mastitic animal milk has a pH value above 7. The pH value is mainly useful in detecting mastitis.

Indicator paper strips are used for measuring pH. A pH value above 6.9 indicates suspicious nature of the sample.

(8) Clot on Boiling Test—The test is a measure of developed acidity. Take 5 ml sample in a test tube and smell for acidity. Keep the tube in a boiling water bath for 5 minutes and smell again. Remove the tube and rotate horizontally to form a film on the sides. Examine for precipitated particles. If a clot is formed then the milk is not suitable. It indicates that lactic acidity is above 0.17% and the milk is not suitable for use.

Note—At high acidity of (0.2% lactic acid) large yellow-brown flakes are observed. At a lesser acidity, a red to lilac colour is seen.

(9) Thickeners—The commonly used thickening agents which help retain the specific gravity of milk after water addition or fat removal, are starch and sugar.

Starch—Take milk sample in a test tube. Add a few drops of iodine solution. Blue colour formed indicates added starch. A distinct colour is obtained if milk serum is used instead of milk.

Cane sugar (Cotton test)—Take 10 ml of milk in a test tube. Add 0.5g of ammonium molybdate and 10 ml dilute HCl. Heat on a water bath to 80°C. An intense blue colour is formed in the presence of sucrose.

(10) **Dirty Water**—Milk does not contain nitrate, as a natural constituent, however, due to added water the same gets introduced and can be detected. Diphenylamine is oxidized by nitrate to a blue coloured complex. Take 5 ml of a milk sample in a test tube. Add 6 drops of mercuric chloride reagent. Add the clear filtrate to 2 ml of diphenylamine reagent. A blue colour formed indicates added water.

(11) **Added Colours**—Buffalo milk is diluted with water, yellow colour is added to it and it is sold as cows' milk. Azo dyes, artificial colours and turmeric are also used for this purpose.

Test 1—Take 10 ml of milk in a test tube, Add an equal volume of ether and shake vigorously. The yellow colour, if added, comes in the ether layer.

Test 2—Take the milk sample in a test tube. Make it alkaline with $NaHCO_3$. Immerse a strip of filter paper in it for half an hour. A yellow or red colour on the filter paper indicates annatto. Treat the paper with $SnCl_2$ reagent. It turns pink if annatto is present.

(12) **Buffalo Milk in Cow Milk (HANSA Test)**—Take a drop of milk on a clean glass slide. Take 15 ml of milk in a test tube. Add 1 ml concentrated HCl and 0.1 g of resorcinol. Mix well and keep in a boiling water bath for 5 minutes. A red colour indicates added sugar.

18.6 ANALYSIS OF BUTTER

In order to test leather the sample is kept in a screw capped jar, and warmed in oven at 32-35°C. It is shaken vigorously till homogenous. It can then be tested the following:

18.6.1 Moisture

The sample is dried in an air-oven at 100-110°C. Weigh accurately, about 3-4 g of the prepared sample, in a clean and dry dish. Place dish on a steam bath for at least 20 minutes stirring in between, till moisture is not seen at the bottom of the dish. Transfer dish to an oven kept at 100°C and keep for 90 minutes. Cool in a desiccator and weigh. Heat the dish again in an oven for 30 minutes and cool. Repeat the process till a constant weight is obtained. The loss in weight is reported as due to moisture and the percentage of moisture can be calculated from this loss.

18.6.2 Fat

Fat is extracted from a dried sample, using petroleum, hydrocarbon solvent. The solvent is removed and fat extracted is weighed. Melt the residue obtained from moisture determination. Add 25 to 50 ml of petroleum hydrocarbon solvent (BP 40-60°C). Filter the solution. Macerate the remaining sediment twice, with solvent and again filter. Collect filtrate in a dried and tared 250 ml flat bottomed flask containing solvent. Wash the filter paper free from fat and collect the filtrate in a flask. Dry the filter paper in an oven at 100°C for 1 hour, till constant weight. The weight of the residue represents the fat in the sample.

18.6.3 Added Salt

The butter is melted in hot water. The soluble chloride in the mixture is titrated with 0.1N $AgNO_3$ solution by Mohr's method.

Weigh accurately about 5g of the sample in a 250ml conical flask. Add carefully 100 ml of boiling distilled water. Keep for 5 to 10 minutes with occasional shaking. Bring the temperature to 50-55°C and add 2ml of K_2CrO_4 indicator solution. Add about 0.25 g $CaCO_3$ and mix by swirling. Titrate the solution against 0.1N $AgNO_3$ till a brownish precipitate is obtained. Carry out a reagent blank identically.

Calculate percent of NaCl as equal to $\dfrac{5.85 \times N \times V}{W}$

Where V = Titration reading , N = Normality, W = Wt. of Sample

18.6.4 Total Titratable Acidity

The titratable acidity is due to hydrolyzed free fatty acids. The value is a measure of the freshness of the sample. Weigh accurately about 18 g of the sample. Dissolve it in 100 ml of boiled water. Titrate with 0.02M NaOH using phenolphthalein indicator.

Calculate acidity as percent Lactic acid = $\dfrac{V}{100}$

Where V = Titration reading.

18.6.5 Added Boric Acid (Preservative)

Melt 10 g of the sample and remove upper fatty layer. To the aqueous layer add 3 drops of saturated oxalic acid solution. Then add 2 drops of 5M HCl and alcoholic turmeric extract. Evaporate the solution over a water bath. A reddish residue indicates added borates.

18.6.6 Test for Oleomargarine and Renovated Butter

The test is based upon the foaming property of pure butter when heated.

Heat about 3 g of the sample over small flame in a spoon. If it is a true butter, copious foam is obtained with clear supernatant fat. If not, then little or no foaming with bumping and sputtering is observed.

18.6.7 Added Colour

The test is based upon colour reactions of dyes with acids/alkalis.

Dissolve the fat separated in fat estimation, in ether. Take two separate aliquots. To one add 1-2 ml of 1:1 HCl and to the other 1-2 ml of 10% NaOH. In the presence of azo dyes acidic solution turns red. If annatto or vegetable colour is present the alkaline tube shows a yellow colour. Other analysis carried out are determination of curd, Cu, Fe, diacetyl, yeast and mould count.

18.7 TESTS FOR ADULTERANTS IN GHEE/BUTTER

(1) Added Vegetable Fat—Badovin test, Halphen test and nitric acid test can be employed. The nitric acid test is based on the fact that the nitro compounds of fatty acid esters are colourless for ghee and butter; but are of different colours in other fats.

Deep yellow - vegetable fat.

Orange - animal fat.

Reddish brown - wax.

Take 3 ml of melted fat in a test tube. Add 3 drops of colourless concentrated HNO_3. Mix well and keep in a hot water bath. Note the colouration of the solution.

(2) Animal Fat—

1. *Nitric acid test* as given above can be employed.

2. *Soda ash test*—Only pure ghee/butter solidifies slowly with Na_2CO_3 Solution other fats do not. Mix equal volumes of melted fat and saturated Na_2CO_3 in a test tube. Keep in a boiling water bath. If turbidity due to soap formation is observed then other fat is present.

3. *Valenta test*—Mix equal volumes of glacial acetic acid and melted sample in a test tube. Keep in warm water. Shake the tube and note the melting temperature for fat. Pure ghee/butter melts between 29-39°C. If melts at a higher temperature then animal fat is present.

(3) Added Non-fat Material

1. *Added starch*—Take the sample in a test tube. Add hot water and mix well. To the aqueous portion add I_2 solution. A blue colour is formed if starch is present.

2. *Added alkai*—To neutralize the acidity and rancidity developed on storage, alkali is added. Take 10 ml of melted sample and transfer to a separator. Add 20 ml of hot distilled water and mix well. To the aqueous layer add drops of phenolphthalein. A pink colour formed indicates added alkali.

Other tests carried out are:

1. Test for phytosterol acetate.

2. DMG test for Ni (from added vanaspati)

18.8 ANALYSIS OF ICE CREAM

For preparing the sample for analysis, let it soften at room temperature. Mix thoroughly by stirring with spoon or by pouring back and forth between two beakers.

18.8.1 Over Run

Over run (increase in volume) of ice cream results because of the air whipped in during the freezing process. Note the volume of ice cream in a given weight mentioned on the carton label. Weigh an empty beaker. Transfer the entire sample in the beaker. Mix the sample and melt at room temperature. Re-weigh the beaker. Calculate the weight of the melted ice cream. Pour melted sample into a clean, dry measuring cylinder and note the volume in ml of melted ice cream. Calculate over run using the formula:

$$= \frac{V_1 - V_2}{V_2} \times 100$$

$$\text{percentage over run (by volume)} = \frac{V_1}{V_2} \times 100$$

Where V_1 = Volume of ice cream; V_2 Volume of melted ice cream.

If the original volume is not known, calculate as follows:

$$\text{Specific gravity } (SG) \text{ of mix} = \frac{W}{V}$$

Where W =   Wt of ice cream.

          V =   Volume of melted ice cream.

Over run = SG * 100

18.8.2 Total Solids

Total solids are reported as the residue remaining upon oven drying. Ice cream is mixed well in a dish containing sand, so that a larger surface area becomes available.

Weigh accurately about 3g of ice cream into a tared, prepared dish containing sand. Wet with a few drops of distilled water. Stir the sand with a glass rod to mix the ice cream properly. Keep the dish on a boiling water bath for 20-30 minutes. Transfer it to an air-oven with a glass rod and heat at 100°C for four hours. Cool in a desiccator and weigh. Repeat till constant weights are obtained. Calculate the loss in weight for ice cream and the percentage of total solids left behind.

$$\text{percentage total solids} = \frac{W_1 - W_2}{W_3} \times 100$$

Where W_1 =   Wt. of dish with sample.

         W_2 =   Wt. of dish with residue.

         W_3 =   Wt. of sample.

18.8.3 Fat (Rose Gottlieb Method)

The ice cream sample is treated with ethanol and ammonia. The separated fat is extracted with a combination of mixed ethers. Ammonia helps soften the curd of milk and alcohol breaks the milk emulsion and separates fat from proteins. It also enhances contact between solvent and fat. The petroleum ether used reduces solubility of alcohol and solvent ether in water. The extracted fat is weighed. The extraction is carried out in a Mojoinner tube. Ammonia also neutralizes the free acidity of the sample which would get ether extracted, the ammonium salts being ether-insoluble. The petroleum ether decreases the solubility of milk, sugar and other non-fat solids soluble in ether. The Mojoinner flask consists of 2 bulbs, a large mixing bulb and a smaller setting bulb, the two being joined by a constriction (Fig 18.2).

Weigh accurately about 4-5g of the prepared sample into an extraction tube. Wash sides of the tube with 2 ml hot water and mix by gentle swirling. Add 2 ml concentrated ammonia and mix thoroughly. Heat on a water bath for 20 minutes at 60°C with occasional shaking. Add 10 ml alcohol and mix. Transfer mixture to a separating funnel. In a beaker rinse 25 ml ether and 5 ml petroleum ether and add to the funnel.

Shake well after each reagent addition for 5 minutes, till a clear upper layer is obtained. Transfer ether layer into a tared flask. Wash extraction tube with a 1:1 mix of solvents and add to the flask. Re-extract the liquid in a separating funnel twice 15 ml of each solvent each time and collect in a tared flask. Evaporate the solvent on a hot plate or a steam bath at 60°C. Dry the residue of fat to constant weight in an oven at 100°C. Cool and weigh the flask Remove fat in the flask with 15-20 ml petroleum ether, dry and weigh as before.

Loss in wt. of flask = wt. of fat

$$\text{Percent fat} = \frac{100\left[\left(\text{Wt. of flask} + \text{dry fat}\right) - \left(\text{Wt. of empty flask}\right)\right]}{\text{Wt. of sample}}$$
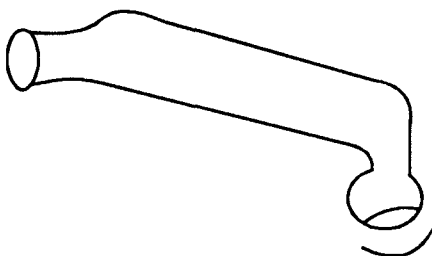
Fig. 18.2 : Mojoinner extraction tube.
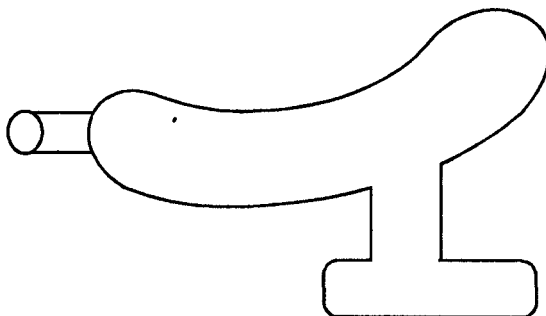
Fig. 18.3 : Jacob's Singer separatory flask.

18.8.4 ACIDITY

The free fatty acids are neutralized with standard alkali. The amount of alkali required is the measure of FFA or acidity.

Weigh 20g of prepared sample in a conical flask. Add 50 ml of recently boiled, cooled water. Titrate the solution with 0.1N NaOH using phenohphthalein indicator. (Rosaniline acetate colour can be employed to judge the end point).

Express the results as percent lactic acid.

$$\text{percentage acidity} = \frac{V \times N}{W}$$

Where V = Volume of alkali, N = Normality

W = Wt. of sample taken

18.8.5 Reducing Sugars

Reducing sugars are determined before and after the inversion by Lane Eynons method. Weigh accurately about 10 g of prepared ice cream sample by the difference method. Transfer it into a 250 ml conical flask and add 150 ml distilled water. Mix well and add neutral lead acetate drop wise, till no precipitate is formed. Add one drop of alumina cream

(clarifying agent), and keep for a few minutes. Rotate in between to ensure protein precipitation. Add enough Sodium-oxalate solution to precipitate excess lead. Filter through fluted No.1 filter paper in a 250 ml flask.

Wash precipitate with hot water. Cool the flask and dilute to 250 ml and titrate by Lane Eynons method.

Inversion—Take 50 ml of the sample solution in a 200 ml graduated flask. Add 25 ml of water and 10 ml of 6.34N HCl and invert. Determine the total invert sugar by Lane Eynons method.

The microbial tests carried out are total colony count and coliform count.

18.9 ANALYSIS OF CHEESE

For preparation of the sample, pass it through a grater or a mortar, transfer it to a sample container. Alternatively cut the sample into small pieces using a sharp knife.

18.9.1 Moisture

Sample is oven-dried with sand at 100°C and loss in weight is reported as percentage of moisture.

Weigh accurately about 3g of the prepared cheese sample in a prepared and tared dish containing sand. Add a few drops of distilled water to the sand. Mix with a glass rod and spread uniformly over the bottom of the dish. Place the dish on a boiling water bath for 20-30 minutes. Transfer with a glass rod to an oven at 100°C and heat for 4 hours. Cool and weigh the dish. Reheat for a period of 1 hour. Cool and weigh again. Repeat the re-heating process till constant weight obtained. Calculate the loss in weight and report as percentage moisture.

18.9.2 Fat (Werner Schmid Process)

The sample is heated with HCl which destroys proteins in the sample. The fat separates on top of the acid (liquid) as a layer. This fat is extracted with mixed ethers (diethyl ether and light petroleum mixture).

Weigh 1 to 1.5 g sample accurately and transfer into Mojoinner extraction tube. Add 10 ml HCl and boil gently with shaking in a boiling water bath till the sample dissolves completely. Cool under a water tap. Add 10 ml alcohol and mix. Ensure proper mixing at every stage. Transfer the solution into a separating funnel. Rinse a beaker with 25 ml ether and 25 ml petroleum ether and transfer the rinsings to a separating funnel. Mix well after each addition, shake vigorously for 5 minutes, keep till a clear upper layer is obtained. Decant ether layer into a tared flask. Rinse extraction tube with 1:1 mixture of ethers and add to the flask. Extract liquid in a separator with 15 ml each of ethers, each time adding water if necessary (omit rinsing with mixed solvents after final extraction). Evaporate solvent in the flask on a hot plate or a steam bath at a temperature of 60°C. Dry to constant weight in an oven at 100°C. Cool and weigh the flask. Dissolve and remove fat in a flask with 15-20 ml petroleum ether. Dry and re-weigh the empty flasks. Calculate loss in weight and report it as percentage of fat.

$$\text{percent fat} = \frac{W_1 - W_2}{W_3}$$

Where W_1 = Wt. of flask and dry fat

W_2 = Wt. of empty flask

W_3 = Sample weight

18.9.3 Added Salt

The fat-free residue from moisture determination is extracted with hot water, to dissolve the salt. Excess $AgNO_3$ is added to it to precipitate the chlorides. The excess is back titrated with KCNS using ferroin as an indicator.

Weigh accurately about 2 g of the prepared cheese sample in a flask. Add 10 ml of distilled water and 25 ml of 0.05N $AgNO_3$ solution. Warm the solution to 80°C. Add 10 ml of concentrated HNO_3 and boil gently (curd digestion) for 10 minutes. At the end of this AgCl becomes granular, liquid of clear lemon yellow colour and fat layer is free from solid material.

Add 2 ml ferroin indicator solution and 50 ml of distilled water. Titrate the solution with 0.05N KCNS, till an orange red tinge is obtained, which persists for 15 seconds. Similarly, titrate 25 ml $AgNO_3$ solution Versus KCNS solution. Determine the amount of $AgNO_3$, consumed from the difference between the two readings. Calculate percent salt as:

$$\text{salt percent} = \frac{0.292 \times V}{W}$$

Where V = Titration reading

W = Wt. of sample taken

18.9.4 Acidity

The acidity due to FFA, is titrated with 0.1M NaOH, and the result is represented as the amount of lactic acid.

Weigh about 20 g of the prepared sample accurately. Macerate it with 200 ml of warm water. Filter the solution and titrate 25 ml filtrate with 0.1N NaOH using phenlophthalein indicator. Calculate the percentage of lactic acid using the relationship 1 ml 0.1N NaOH = 0.0090g of lactic acid.

18.10 COMMON ADULTERANTS

Milk—Buffalo milk sold as cow milk after dilution or buffalo milk added to cows' milk, fat removal, coal tar dyes, starch of potato and sweet potato origin, water.

Ice Cream—Non permitted additives like colourants, flavours and sweeteners, starch, deficiency in milk fat and total solids content.

Butter/Ghee—Vanaspati, animal fat, excess moisture, non-permitted colours, starch, rancid fat.

□□□

19

ROUTINE MICROBIOLOGICAL EXAMINATION OF MILK

19.1 INTRODUCTION

Milk, if allowed to stand for sometime at room temperature, will develop an enormous number of microorganisms through multiplication of those previously introduced. The number of microorganisms in milk at the time when it is consumed is determined by the following factors (1) the amount of initial inoculum received in milking and handling, (2) the time interval between production and consumption, during which the organisms have multiplied, (3) the temperature at which the milk was held, and (4) whether the milk was pasteurized. Microbiological and chemical testing of milk is carried out to determine whether it has been produced and handled under proper sanitary conditioning to preserve it from spoilage. High microbial counts do not necessarily mean that disease-producing organisms are present. However, it does indicate excessive contamination or improper cooling, and holding conditions. The methods commonly employed for enumerating microbial population of milk are as follows (1) the standard plate count (SPC), (2) the direct microscopic count (DMC), and (3) the reductase test.

19.2 THE STANDARD PLATE COUNT (SPC)

This is an agar plate method and is commonly used for the estimation of the number of bacteria in milk. A series of dilutions of milk sample is prepared. 1 ml of diluted sample is mixed with melted agar cooled to 45°C, and the inoculated medium is then poured into the plate. Plates may be incubated at 32 to 35°C for 48 hours for routine work, at 7°C for 7 to 10 days to enumerate psychrophiles, or at 55°C for 48 hours to enumerate thermophiles. The visible colonies are counted and the number of colonies per millilitre for the milk sample is reported as the, standard plate count per millilitre.

A protocol for SPC of milk is outlined in Figure 19.1.

The factors which influence plate counts include temperature of incubation, period of incubation, composition of plating medium, amount of oxygen, etc. Unless a standard procedure is followed, the results from different laboratories may show wide variation.

Advantages of the plate method—The agar plate method is especially suited to determinations where bacterial numbers are low. The method is generally used for testing pasteurized milk and the higher-grade raw milk. It is the only method approved by the American Association of Medical Milk Commissions for examining samples of certified milk.

Objections to the plate method—Objections to the method are numerous. The most important are probably the following: Pathogenic bacteria usually are not detected. It they grow, they cannot be distinguished from non-pathogenic forms by appearance. The number of colonies appearing on agar do not represent all organisms present in the milk. Many of the organisms fail to develop. Anaerobic organisms do not find conditions favourable for growth. This means that no single medium or given set of conditions is capable of giving growth of all viable organisms likely to be found in milk. A temperature of $32 \pm 1°C$ is not the optimum for the growth of all organisms. Shaking the sample does not break up all clumps or groups of bacteria. Chains of streptococci usually remain intact and record as only one colony. The colony counts represent only a fraction of the total bacterial content of milk. Agar counts should be regarded as estimates rather than exact numbers.

<div align="center">

Take 1 ml of well mixed sample
in 9 ml phosphate buffer in a tube

↓

From this 1:10 diluted milk prepare
required number of further serial
dilutions in the similar way.

↓

Pour 1 ml diluted sample from atleast
2 appropriate dilutions in petridishes.

↓

Add 15-20 ml of melted and cooled (45°C)
Standard Plate Count Agar (SPCA) in the
plates and mix gently but thoroughly

↓

Allow the plate to solidify

↓

Incubate at 37°C for 48 h for routine work

(at 7°C for 7 days to enumerate
psychrophiles, or at 55°C for 48 hours
to count thermophiles)

↓

Count the number of colonies and
express as c.f.u/ml by multiplying
it with the dilution factor.

</div>

Fig. 19.1 : Procedure for Standard plate count of milk.

Because of the long incubation period, the milk is usually consumed before information on the number of bacteria present is available. However, the quality of a milk supply cannot be determined on the basis of one bacterial count. A series of counts is necessary to establish the quality of the milk source.

19.3 BREED COUNTING METHOD/DIRECT MICROSCOPIC COUNT-DMC

This is a direct count method in a known area of microscopic field. The milk sample is spread (about 0.01 ml) over 1 cm^2 of a microscopic slide. Milk smear is dried and stained with Newman-Lampert stain. This stain fixes the smear, dissolves fat globules and stains bacteria with Methylene blue. Slide is then scanned under several oil immersion microscopic fields.

Calculations are made as follows:

Area of one microscopic field = 0.02 mm^2

Area over which milk sample is spread on the slide = 1 cm^2 or 100 mm^2

Then, no. of field possible under oil-immersion lens

= 100 mm^2/ 0.02 mm^2

= 5,000 microscopic fields

This number of fields is for 0.01 ml of milk sample. However, final count is expressed as bacterial number per ml of sample. Thus, factor is to be multiplied by 100 i.e. 5000 × 100 = 5,00,000

This is the microscope factor i.e. 5,00,000 or 5.0×10^5. Supppose, 20 fields yielded an average of 2 bacteria per field. Then, the final count would be

2.0 × 5,00,000 (microscope factor)

= 10,00,000 bacteria per ml of milk sample.

Limitations—

(1) This method does not distinguish between live and dead cells.

(2) The microscopic method may be the source of considerable error unless the milk contains a high count. This is because a large factor is used for converting the number of organisms per field to the number per millilitre of milk.

Advantages—

(1) Results can be obtained quickly, usually in 10 minutes.

(2) Since less work is required, more samples can be examined by this method than by the plate method.

(3) The amount of equipments and glasswares necessary are much less.

(4) The slides may be preserved as a permanent record and examined whenever occasion aries, whereas plates must be examined and discarded.

(5) Some idea of the morphological types can be obtained from slide preparations. This is frequently of great value in determining the cause of the bacterial count and nature of the contamination.

(6) If microscopic examination reveals an excessive presence of leucocytes and other body cells in milk, it indicates a diseased condition of udder.

Some believe that the method is less useful for pasteurized milk because most of the bacteria are dead. This is not necessarily so. It has been shown that bacteria killed by heat

treatment usually disintegrate and gradually lose their ability to stain within a few hours after pasteurization. Even if the bacteria remain intact, they are usually distinguished from living forms by their inability to stain intensely.

Comparison of counts by the two methods—The microscopic method gives much higher individual cell counts than the agar method. The differences between the counts by the two methods are considerably greater on samples showing low bacterial counts than on those showing high counts. The organisms in low-count milk generally represent external contaminants that fail to develop on agar, whereas those organisms in high-count milk are forms which have developed in the milk. Also, low-count milk usually shows a greater percentage of clumps than high-count milk.

Bacterial standards are generally based on microscopic clump counts or agar plate counts, the standards being the same by either method. However, if individual cells are counted, the slide method gives higher results, the ratio being 3.33:1. Therefore total cell counts by the slide method are approximately 3.33 times greater than counts by the plate method. The word 'clumps refers' to isolated cells as well as to groups of bacteria.

Direct Epifluorescent Filter Technique (DEFT)—A modern technique

DEFT is widely explored in food and dairy industry for counting bacteria. This technique is summarized in following steps:

A. *Membrane filtration*
  1. Add 2 ml milk to 0.5 ml crude trypsin
  2. Add 2 ml Triton X-100 (0.5%)
  3. Incubate at 50°C for 10 min
  4. Pre-warm 0.6 μm pore size Nucleopore membrane filter and holder
  5. Filter-treated milk
  6. Rinse reaction tube and filter tower
B. *Staining*
  7. Overlay filter with 25 ml acridine orange/Tinopal *(0.025%)* for 5 min
  8. Rinse with 2.5 ml pH 3 buffer
  9. Rinse with 2.5 ml ethanol (95%)
  10. Mount membrane in immersion oil
  11. Examine by epifluorescent microscopy

19.4 DYE REDUCTION TESTS (REDUCTASE TESTS)

Milk, after it leaves the animal, becomes exposed and mixed with air and acquires an oxidation-reduction potential (O/R) of about +300 mv. As organisms grow in milk, they cause a lowered (O/R) potential. The rate of shift in O/R potential depends on the number' and kinds of organisms and the rate of their metabolism. The shift, can be detected by the use of various dyes (methylene blue, resazurin) which undergo colour changes when reduced or oxidized. It should be, remembered that some redox reactions involve oxygen and some do not. Many redox reactions involve electron transfer. One reactant must serve as the electron donor and become oxidized, and the other reactant must serve as the electron acceptor and become reduced. Therefore, the rate at which a dye (electron or H acceptor) is reduced can be interpreted as the rate at which the substrate (milk) is oxidized by living organisms.

19.4.1 Methylene Blue Reduction Test (MBRT)

Principle—The method depends upon the ability of bacteria in milk to grow and to consume the dissolved oxygen, which reduces the oxidation-reduction potential in the medium. Certain enzymes present in bacteria, known as oxidoreductases, are then able to produce oxidations by the removal of hydrogen. The methylene blue accepts the hydrogen and becomes reduced to the colourless or leuco compound. The speed of decolouration is an indication of the rate at which oxidation takes place.

Methylene blue is of value in making a rapid survey of the quality of raw milk. The rate of decolourization depends upon the number of organisms present. The test can be employed to determine, in a rough way, the bacterial population of a milk sample. The procedure is quickly and easily carried out and with a minimum of expense. It is particularly valuable in making rapid inspections of large numbers of samples to determine whether the milk received by companies answers the requirements prescribed by law.

Procedure—The test is expressed as the time required for a known concentration (about 1:250,000) of methylene blue thiocyanate to lose its colour when the milk is incubated at 35 to 37°C to hasten oxygen consumption and to shorten the period of observation. Under some conditions the blue colour does not disappear uniformly. In such cases the end point is taken as the time required for the milk to show no blue colour after it is mixed. A protocol for MBRT is shown in Fig. 19.2.

<p style="text-align:center">Thoroughly mix the milk sample</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Take 10 ml milk sample in sterilised standard test tube</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Add 1 ml standard Methylene blue solution</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Replace cotton plug of the tube with sterile rubber bung</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Mix dye & milk by inverting the tube twice</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Place tubes in water bath at 37°C</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Observe tubes after every 30 min.</p>
<p style="text-align:center">If there are no signs of reduction, invert the tube and incubate further. If decolourization has commenced, the tube should not be inverted.</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Continue observation till complete decolourization is achieved</p>
<p style="text-align:center">↓</p>
<p style="text-align:center">Record the time taken for decolourization in hours as MBR time.</p>

Fig. 19.2 : Methylene blue reduction test.

Limitation—There is not always good agreement between the methylene blue reduction time and the agar plate count because (1) some organisms fail to grow on nutrient agar; (2) a clump of organisms records as only one colony, whereas the rate of decolourization is due to the combined effect of each member of the mass; (3) the rate of decolourization of the stain is not the same for all organisms; and (4) the test becomes less accurate as the

reduction time is increased, freshly drawn milk requiring at least 10 hr to decolorize methyhene blue.

Grading—Milk may be classified on the basis of the methylene blue reduction time as follows:

Class 1—Excellent milk, not decoulorized in 8 hr.

Class 2—Good milk, decolorized in less than 8 hr, but not less than 6 hr.

Class 3—Fair milk, decolorized in less than 6 hr, but not less than 2 hr.

Class 4—Poor milk, decolorized in less than 2 hr.

The reduction time limit for grade A milk to be pasteurized is 5½ hr; for milk to be consumed raw, 8 hr; for raw cream to be pasteurized, 5 hr; and for cream to be consumed raw, 7 hr.

19.4.2 Resazurin Test

Principle—As milk for pasteurization improves in quality, the methylene blue reduction time increases. This requires incubation for inconveniently long periods. To provide comparable information in less time, the resazurin test is recommended as a substitute for the methylene blue reduction test.

Resazurin (diazoresorcinol) is an oxidation-reduction indicator having a pH range of 6.5 to 3.8. At pH 6.5 and above, the indicator is purple in colour; at pH 3.8 and below, it is pink. A gradual colour change occurs between these two extremes.

On complete reduction of resazurin to a pink colour, the compound resorufin is formed in milk. This reaction is irreversible. In the second stage, the resorufin is further reduced to dihydroresorufin, followed by a gradual disappearance of the pink colour. This reaction is reversible.

The indicator is capable of being reduced by bacteria. The greater the bacterial count, the faster the stain is reduced. Because of this fact, it may be used to estimate the number of bacteria in milk. The test provides results in less time than by the methylene blue reduction method. Since resazurin is also sensitive to the reducing action of leucocytes, it reveals the presence of the udder disease known as mastitis

Procedure—The test is performed as follows: The milk sample is thoroughly mixed and 10 ml pipetted into a 5/8- by 6-in, test tube. One ml of a 1:20,000 dilution of resazurin is added to the milk and the tube is inverted three times slowly to mix well. The tube is placed in a 35 to 37°C water bath *and* examined at the end of each of three successive hourly intervals.

Interpretations—The colour of the milk should be compared with a series of standard disks prepared for the purpose. If the colour remains blue or lilac (disks 6 or 5) for 1 hr. the milk is normal in bacterial content. If mauve or mauve-pink (disk less than 5), the bacterial content is high and the milk should be regarded as abnormal. If a disk number less than 3 is obtained in 1/2 hr, the milk is grossly abnormal. If the stain is completely reduced (disk O) in 1 hr or less, the milk contains many pus cells and is probably teeming with mastitis organisms.

19.5 TESTS FOR DETERMINING BACTERIOLOGICAL QUALITY OF DIFFERENT DAIRY PRODUCTS

The proposed bacteriological tests to be carried out on different dairy products are summarised in Table 19.1.

Suggested microbiological standards for different dairy products are shown in Table: 19.2.

Table 19.1 : Proposed tests for determining the bacteriological quality of different dairy products

Sample	Sample size	Test or count	
		When carried out	**Type of test**
Raw milk	Not less than 200 ml or unopened retail container	On receipt (held at 0-5°C until tested, but not longer than 24h) and/or after storage at specified temperatures, e.g. 7°C When testing for antibiotics maintain sample at –30 to –15°C to minimise inactivation of penicillin	Total bacteria Direct microscopic count or plate count Coliforms *(E. coli)* Psychrotrophs Proteolytic or lipolytic organisms Thermoduric bacteria Spores *(B. cereus)* Dye reduction (e.g. methylene blue, resazurin test) Antibiotics Pathogens (if required) Shelf-life
Cream (raw or pasteurised)	See 'milk'	See 'milk'	See 'milk'
Cultured dairy products. e.g. yoghurts, cultured milks, sour creams	Unopened consumer pack to give a minimum sample of 100 g (bulk containers are sampled in the field)	On receipt (held at 0—5°C until tested, but not longer than 24h)	Direct microscopic examination of the types of bacteria in reference to the culture expected Coliforms *E. coli* Psychrotrophs Yeasts and moulds

Sample	Sample size	Test or count	
		When carried out	Type of test
			Staphylococci (coagulase–positive)
			Viable culture organisms
			pH
			Shelf-life
Sweetened condensed milk	Not less than 200g or unopened retail container	One set: on receipt Another set: after 1 month at 25°C	Total bacteria (plate count or direct (microscopic count)
			Yeasts (low level contamination)
			Moulds (microscopic examination of lumps for mould mycelia)
			Micrococci
			Staphylococci coagulase--positive)
			Thermoduric organisms
Canned dairy products (sterile), e.g. evaporated milk, flavoured milk. cream in cans	Intact retail can	One set: on receipt Another set: after 1 month at 25°C	Total bacteria (plate count or direct microscopic count)
			Anaerobic spores
			Sterility test
			pH
UHT milk UHT cream UHT products	Two or three sets of containers (A and B or A, B and C)	A: on receipt B: after 5-7 days at 35-37°C *or*	Direct microscopic examination (if suspected of being non-sterile)

Sample	Sample size	Test or count	
		When carried out	Type of test
Sterilised milk		A: on receipt	Sterility test
		B: after 7 days at 33°C	
		C: after 7 days at 55°C	
Cheese	50-200 g or small wrapped	On receipt (held at 0-5°C	Coliforms
Cheese spreads	domestic portion	until tested)	*E. coli*
Cheese powders			Yeasts and moulds
			Staphylococci (coagulase. positive)
			Contaminating organisms
			Other pathogens (as required)
Butter	50-200g or small packs to	On receipt (held at 0-5°C	Total bacteria (plate count)
Butter products	yield at least 50g	until tested)	Contaminating organisms
			Yeasts and moulds
			Lipolytic bacteria
			Proteolytic bacteria
			Coliforms *(E. coil)*
			Psychrotrophs
			Staphylococci (coagulase- positive)
Dried milk	50-500 g	On receipt (held at 0-5°C	Total bacteria (direct
Dried dairy products, i.e.		until tested)	microscopic count and
buttermilk powder, whey			plate count)
powder, ice cream mix			Coliforms

Sample	Sample size	Test or count	
		When carried out	**Type of test**
powder			Thermophilic bacteria
			Spores
			Staphylococci (coagulase-- positive) --
			Salmonellae
			Antibiotics
Casein	50-500g	On receipt (held at	Total bacteria (plate count)
Caseinates		temperatures below 25°C	Coliforms
Coprecipitates		until tested)	Thermophilic bacteria
			Yeasts and moulds
			Staphylococci (coagulase-- positive)
			Salmonellae
Ice cream	At least 50g	Stored at a temperature not	Total bacteria (plate count)
Frozen milk products		exceeding –15°C until	Psychrotrophs
		tested	Coliforms
			E. coli
			Yeasts and moulds
			Staphylococci (coagulase- positive)
			Salmonellae

Table 19.2 : Suggested microbiological standards for different dairy products (figures in parentheses mean 'aimed at')

Product	Test	Count or result
Raw milk for liquid consumption after being pasteurised	Total bacterial count	<250000 (50000) ml^{-1}
	Coliforms	<100
	E. coli (faecal type)	Absent in 0.01 ml
	Thermoduric count	$<1\,000\ ml^{-1}$
	Spores	$<10\ m^{-1}$
	B. cereus (spores)	$<1\ ml^{-1}$
	Staph. aureus (coagulase-positive)	<100 (10)
	Methylene blue reduction time (at 37°C)	Not less than 5 h
	3-h Resazurin test (at 37°C, Lovibond disc reading)	Not less than 3h
	Somatic cell count	$<750000\ ml^{-1}$
Raw milk	Total bacterial count	<50000 (10000) ml^{-1}
Raw cream (to be consumed raw)	Coliforms	$<10\ ml^{-1}$
	E. coli (faecal type)	Absent in 1 ml
	Methylene blue reduction time (at 37°C)	Not less than 7 h
	3-h Resazurin test (at 37°C Lovibond disc reading)	Not less than 4 h
	Staph. aureus (coagulase-positive)	$<10\ ml^{-1}$
	Somatic cell count	$<500000\ ml^{-1}$
Pasteurised market milk	Total bacterial count	<50000 (5000) ml^{-1}
Pasteurised cream	Coliforms (after processing)	<1 (0.1) ml^{-1}
	E. coli (faecal type)	Absent in 10 ml
Dried milk	Direct microscopic clump count	<50000000 (10000000) g^{-1}
Dried milk products	Total bacterial count (plate Count)	<100000 (50000) g^{-1}
	Yeast and moulds	$<10\ g^{-1}$
	Coliforms	Absent in 1 g

contd. ...

Product	Test	Count or result
	E. coli. (faecal type)	Absent in 10 g
	Staph. Aureus (coagulase-postive)	Absent in 1 g
	Salmonellae	Absent in 25 (100) g
Ice cream	Total count (plate count)	<50000 (5000) g
	Coliforms	<10 g^{-1}
	Staph. aureus (coagulase-positive)	<10 g^{-1}
	E. coli. (faecal type)	Absent in 1g
	Salmonellae	Absent in 25(100) g
Cultured milks	Yeasts and moulds	<10 (1) g^{-1}
Cultured cream	Coliforms	<10 (1) g^{-1}
Sweetened condensed milk	Total count	<1000 (100) g^{-1}
	Yeasts and moulds	<1 g^{-1}
	Coliforms	<10 (1) g^{-1}
Butter	Contaminating organisms (non-lactic acid bacteria)	<50000 (10000) g^{-1}
	Proteolytic organisms	<1000 g^{-1}
	Lipolytic organisms	<1000 g^{-1}
	Yeasts and moulds	< 10 g^{-1}
	Coliforms	< 10(1) g^{-1}
	E. coli (faecal type)	Absent in 1 g
Cheese	E.. coli (faecal- type)	Absent in 0.01 g
	Staph. aureus (coagulase-positive) (at 1 month)	Absent in 0.1 g
Cottage cheese	Yeasts and moulds	<10 (1) g^{-1}
	Coliforms	<10 g^{-1}
Casein	Coliforms	<10 g^{-1}

19.6 COMPOSITION OF MICROBIOLOGICAL MEDIA AND REAGENTS USED IN DAIRY INDUSTRY

1. Phosphate buffer (Used *for* dilution of samples)

KH_2PO_4	:	34.0 g
Distilled water	:	1000 ml
pH	:	7.2 (to be adjusted by 1N NaOH)

For use as dilution water, take 1.25 ml and make up to 1 lit by distilled water.

2. Newman Lampert Stain (Used for Direct Microscopic Count)

Methylene blue powder	:	0.6 g
Ethyl or Absolute alcohol	:	52 ml
Tetrachloroethane	:	44 ml
Glacial acetic acid	:	4 ml

Mix ethyl alcohol and tetrachloroethane, warm it and add methylne bule powder. Lastly slowly add acetic acid and filter.

3. Standard Plate Count Agar (SPCA) (Used for enumeration of total viable bacteria)

Tryptone	:	5.0 g
Yeast extract	:	2.5 g
Glucose (dextrose)	:	1.0 g
Agar (bacteriological grade)	:	15.0 g
Distilled water	:	1000 ml
pH	.	7.0± 0.1

4. Milk agar (Used for enumeration of total bacteria, spores or proteolytic bacteria)

Peptone	:	5.0 g
Yeastrel	:	3.0 g
Agar	:	15.0 g
Fresh milk	:	10.0 ml
Distilled water	:	1000 ml
pH	:	7.0± 0.1

5. Violet Red Bile Agar (Used for enumeration of coliforms)

Yeast extract	:	3.0 g
Peptone or gelysate	:	7.0 g
Sodium chloride	:	5.0 g
Bile salt No.3	:	1.5 g
Lactose	:	10.0 g
Neutral red	:	0.03 g
Crystal violet	:	0.002 g
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	7.4±0.2

6. MRS agar (Used for enumeration of Lactobacilli)

Peptone	:	10.0 g
Beef or meat extract	:	10.0 g
Yeast extract	:	5.0 g
Glucose	:	20.0 g
Tween 80	:	1.0 ml
K_2HPO_4	:	2.0 g
Sodium acetate	:	5.0 g
Trisodium citrate	:	2.0 g
$MgSO_4. 7H_2O$	:	200 mg
$MnSO_4. 4H_2O$	:	50 mg
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.2 ± 0.2

7. Potato Dextrose Agar (Used for enumeration of yeasts and molds)

Infusion from potatoes	:	200 g
Dextrose	:	20 g
Agar	:	20g
Distilled water	:	To make final volume to 1000 ml
pH	:	3.5

The pH should be adjusted by 10% sterile tartaric acid, after sterilization i.e. just before pouring the medium into plates.

8. Lactose Purple Agar (Used for differentiating lactic and non-lactic organisms)

Yeast extract	:	3.0 g
Peptone	:	10.0 g
Acid casein hydrolysate	:	10.0 g
Beef extract	:	3.0 g
Lactose	:	10.0 g
Bromo cersol purple (1% aqueous solution)	:	2.5 ml
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	7.0

9. Milk fat Agar (Used for enumeration of lipolytic organisms)

A. Base medium

Peptone	:	5.0 g
Beef extract	:	3.0 g
Sodium chloride	:	5.0 g
Agar	:	20.0 g

| Distilled water | : | 1000 ml |
| pH | : | 7.2 |

B. Fat solution:

Take appropriate quantity of milk fat in 10% solution of nile blue sulphate or chloride. Shake thoroughly and remove excess dye by washing the fat several times. Finally sterilise the stained fat.

C. Complete medium

Before pouring in plates, add 1 % stained fat (B) in melted and tempered base medium (A) and mix uniformly.

10. Brain Heart Infusion Broth (Used for enrichmet of pathogens)

Peptone	:	10.0 g
Dehydrated calf brain infusion	:	12.5 g
Dehydrated beef heart infusion	:	5.0 g
Glucose	:	2.0 g
NaCl	:	5.0 g
Na_2HPO_4	:	2.5 g
Distilled water	:	1000 ml
pH	:	7.4

11. Baird Parker Agar (Used for selective enumeration of *Staphylococcus aureus*)

A. Base medium

Tryptone	:	10.0 g
Yeast extract	:	1.0 g
Meat extract	:	5.0 g
Glycine	:	12.0 g
Lithium chloride	:	5.0 g
Agar	:	12 to 20 g
Distilled water	:	1000 ml
pH	:	7.2

Sterilise by autoclaving at 121°C for 15 min.

B. Tellurite solution

Dissolve 1 g potassium tellurite in 100 ml distilled water and sterilize by filteration.

C. Sodium pyruvate solution

Dissolve 20 g sodium pyruvate in 100 ml distilled water and sterilize by filteration.

D. Egg-yolk emulsion

Wash the fresh hens' eggs in liquid detergent, rinse and dip in 70% alcohol and drain. Using aseptic procedures break each egg into a petridish and separate yolk. Take it to sterile measuring cylinder with four volumes of sterile water and mix thoroughly. Heat the mixture at 45°C for 2 hours and leave at 0 to 5°C for 18-24 hours. Collect the supernatant emulsion aseptically.

E. **Complete medium**

Base medium (A)	:	90.0 ml
Tellurite solution (B)	:	1.0 ml
Sodium pyruvate solution (C)	:	5.0 ml
Egg-yolk, emulsion (D)	:	5.0 ml

12. Tetrathionate broth (Used for enrichment of Salmonella and Shigella)

Meat extract	:	5.0 g
Peptone	:	10.0 g
NaCL	:	3.0 g
$CaCO_3$	:	45.0 g
Distilled water	:	1000 ml
pH	:	7.0±0.1

13. Xylose Lysine Deoxycholate (XLD) Agar (Used for selective enumeration of Salmonella and Shigella)

Xylose	:	3.5 g
L-lysine	:	5.0 g
Lactose	:	7.5 g
Sucrose	:	7.5 g
NaCl	:	5.0 g
Yeast extract	:	3.0 g
Sodium deoxycholate	:	2.5 g
Sodium thiosulphate	:	6.8 g
Ferric ammonium citrate	:	0.8 g
Phenol red	:	0.08 g
Agar	:	15.0 g
Distilled water	:	1000.0 ml
pH	:	7.4±0.2

14. Meat Liver Agar (Used for selective enumeration of sulphite reducers)

Meat liver base	:	20.00 g
Glucose	:	0.75 g
Starch	:	0.75 g
Sodium sulphite	:	1.20 g
Ferric ammonium citrate	:	0.50 g
Agar	:	11.00 g
Distilled water	:	1000 ml
pH	:	7.6±0.2

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20

HAZARD ANALYSIS
CRITICAL CONTROL POINT—HACCP

20.1 INTRODUCTION

Before 1960, controlling the microbiological quality and safety of manufactured foods and raw materials was based mainly on microbiological testing and inspection. Neither method proved to be entirely satisfactory in achieving the objective of producing safe high quality food. A different, far more systematic approach to safety and quality was formulated in the 1960s. The new system, called the **Hazard Analysis Critical Control Point** method (HACCP), originated in the engineering industry and was developed and applied to food processing as part of the US space programme for the production of zero defect foods for astronauts. It was soon realized that this new approach to producing microbiologically safe food had great potential, and in 1973 HACCP was adopted by the USA Food and Drug Administration for the inspection of low acid canned foods. Since then information about HACCP has been disseminated throughout the food industry and the method widely adopted by both food manufacturers and caterers as a method of controlling food quality.

This chapter briefly explains the implementation of a HACCP program, and provides a model HACCP program as a guide to developing an effective system in a dairy plant.

20.2 Important Elements of HACCP Approach

The following are important elements of the HACCP approach:

(1) **Hazard.** Anything associated with the food that has the potential to cause harm to the consumer or the product. This can mean:

    (a) unacceptable contamination, growth, or survival of a food poisoning organism;

    (b) presence of a microbial toxin;

>   (c)   unacceptable contamination, growth, or survival of a food spoilage organism;
>
>   (d)   presence of a microbial enzyme that can cause spoilage.

A hazard can occur during raw material production, manufacturing, distribution, retailing or consumer use. Not all hazards are microbial.

A hazard may be chemical, such as the presence of pesticide residues or physical, e.g. the presence of a foreign body.

> **(2) Critical Control Points (CCPs).** CCPs are steps or procedures that can be identified during raw material production, manufacturing, retailing, distribution or consumer use in which hazards can be controlled. Identifying CCPs focuses attention on specific prevention and control measures. The ICMSF recognizes two types of CCP: CCP1s will assure the control of a hazard and CCP2s will minimize a hazard but cannot assure its control. Thermal processing of low acid canned foods would be categorized as a CCP1, guaranteeing the safety of the product if carried out correctly. Correct container handling after processing would be a CCP2 because, although this operation is important in reducing the incidence of spoilage and the likelihood of post process recontamination with pathogens, it cannot guarantee that the product is safe. This approach is not universally accepted.
>
> **(3) Risk.** Risk is the likelihood of a hazard occurring. Quantifying the risk of a microbial hazard occurring can be difficult. Risk is often ranked as low, medium or high.
>
> **(4) Severity.** Severity is the level of danger presented by a hazard. A risk can be very low and the severity high, e.g. the risk from bovine tuberculosis in milk produced by the modern dairy industry is extremely low as a result of the attested herd scheme and pasteurization. The severity of the disease if it did occur is, however, high.
>
> **(5) Monitoring.** Monitoring involves checking whether a CCP is under control. Monitoring can involve visual inspection, smell, physical measurement, chemical measurement or microbiological testing. Monitoring should detect any loss of control over a CCP in time for corrective action to be taken before there is a need to reject the product. This means that, because of the time lag involved in producing results, microbiological tests are normally associated with verification rather than monitoring. Exceptions are ATP photometry for hygiene control, DEFT and dye reduction tests. Monitoring is sometimes given a wider interpretation when shelf-stable products, e.g. canned foods, UHT milk and frozen foods, can be held before release until the results of microbiological testing are available.
>
> **(6) Verification.** Verification is the application of tests that are additional to those used for monitoring. Verification determines whether the HACCP system is operating correctly, e.g. the chlorine levels in can-cooling water can be monitored to ensure that the levels of contamination are kept to an acceptable level and do not cause post process recontamination resulting in unacceptable levels of leaker spoilage. Incubation tests can be carried out on cans to verify that post process recontamination has not taken place.

20.3 STAGES IN SETTING UP HACCP

Setting up an HACCP system is a highly complex process requiring a detailed consideration of:

- raw materials;
- each stage of the manufacturing process;
- the distribution system;
- retailing;
- education of the workforce;
- the target consumer;
- the way in which the consumer is likely to handle the product in relation to hazard or risk;
- monitoring and verification.

The main stages involved in setting up an HACCP system for a product are summarized in Fig. 20.1.

Fig. 20.2 shows a flow diagram for the production of bottled, pasteurized milk. CCPs are located when:

- pre-process contamination with pathogen or spoilage organisms can occur;
- post process recontamination with pathogens or spoilage organisms can occur;
- unacceptable growth of spoilage organisms can occur;
- pasteurization is used to kill pathogens.

Not all stages are CCPs, for example, distribution by doorstep delivery is not controlled in relation to the temperature at which the milk is held and is not, therefore, a CCP.

20.4 EXAMPLE OF CCP MONITORING AND VERIFICATION

Milk is a potential hazard with regard to a number of important pathogens, that is, *Mycobacterium bovis, Salmonella, Campylobacter, Listeria monocytogenes* and *Yersinia enterocolitica.* All of these organisms are destroyed by the pasteurization process originally designed to kill *M. bovis.* The minimum heat treatment required by law in the UK is 71.7°C for 15 seconds (high temperature short time—HTST process).

Monitoring

- Temperature in the pasteurizer holding tube.
- Observation of correct plant functioning by trained operators.
- Ensure correct operation of low diversion device at the end of the holding tube; milk not reaching the correct temperature should be returned to the raw milk side.

Verification

- Examination of thermographic records.
- Phosphatase test—phosphatase enzyme in milk is destroyed if pasteurization has been carried out correctly.

Identify HACCP team	HACCP uses a multidisciplinary team e.g. microbiologist, food technologist, food engineer, hygiene specialist
Construct process flow diagram	A detailed flow diagram is constructed showing each stage in the process. An additional plan of the factory layout may help in identifying CCPs
Identify hazards Assess severity or risk	Hazards are identified for each stage of the process, including raw materials in relation to the product formulation, the process, distribution and consumer use
Identify CCPs	This can be the raw material, a specific processing step, product formulation, plant layout and construction, cleaning and disinfection or employee hygiene
Specify criteria for CCPs	A criterion is required for each CCP e.g. for pasteurization the specific time and temperature
Set tolerances for criteria	Upper and lower limits for each CPP criterion are set where appropriate
Establish monitoring system for each CCP	Establish the monitoring procedure for each CPP. This can be visual, sensory, physical, chemical or microbiological
Establish plan for corrective action	Establish what action will be taken if monitoring demonstrates that a CCP is not under control
Establish procedures for verification	Establish procedures to demonstrate that HACCP is working correctly using supplementary tests and information.
Establish system for documentation and recording	HACCP scheme should be fully documented. Records should be kept of monitoring, corrective action, results of verification and employee training
Establish procedure for review	HACCP should be reviewed periodically. Review is essential with any changes in processing equipment, procedures or product formulation

Fig. 20.1 : Setting up an HACCP programme.

Fig. 20.2 : CCPs in the production of bottled milk.

21

SHELF-LIFE PREDICTING METHODS FOR MILK

21.1 INTRODUCTION

The term **"shelf-life"** can be used interchangeably with the term **"Keeping quality,"** which is defined as the time a product remains acceptable in flavour after packaging. The question then becomes, what is an acceptable shelf-life for fluid milk products? Before answering this question, the temperature at which the product is held when shelf-life testing is done must be specified. The temperature most commonly used is 7°C (45°F), which is chosen because it approximates the temperature of dairy cases in supermarkets and the home refrigerator (Bishop and White, 1985; White, 1991). Also, as has previously been pointed out (Bishop and White, 1985), in all shelf-life prediction studies, the "potential" shelf-life is actually what is being measured because the experimental sample stored in a cooler in the laboratory is not subjected to the rigors of distribution and transportation.

Almost all tests that are designed to predict the shelf-life of dairy products are based on detection of gram-negative psychrotrophic bacteria (especially the pseudomonads). These microorganisms cause most shelf-life problems, especially in fluid milk and cottage cheese. Regardless of the method, the key (White, 199]) to predicting the shelf-life of milk and milk products is that the method must be rapid—reliable and meaningful results must be obtained within 72 hours and ideally within 24 hours.

In addition, results of tests to predict shelf-life must be compared or correlated with the actual product shelf-life. Thus, to determine whether or not a particular test to predict shelf-life is effective, the actual product shelf-life must be assessed. The actual product shelf-life is determined by holding the samples at 7°C and testing them every day until an off-flavour develops. The shelf-life is then estimated as the day the off-flavour developed minus one. To minimize the number of times the container is opened and closed, the products do

not need to be tasted until after day 10 (assuming that the product had a shelf-life of 10 days or more). It is important in determining basic product shelf-life to use the same container because each filler head (on a gallon filler) can yield significantly different results. In selecting samples from a filler, it is good to rotate the samples obtained so that, over a given period, all filler heads can be sampled.

Correlation between the results of shelf-life prediction and actual product shelf-life at 7°C can be ranked using the following scale: excellent = >0.90; good = 0.80 to 0.89; fair = 0.70 to 0.79 (Bishop, 1988, 1993; White, 1991). Because of low initial numbers of bacteria in freshly pasteurized milk, most shelf-life testing consists of preincubating the product (in its original container) at 21°C for 18 hours followed by some rapid bacterial detection method (White, 1991, 1993, 1996).

21.2 METHODS

Some of the proven methods to predict shelf-life are as follows:
1. Moseley Keeping Quality Test.
2. PI plus various plating methods: PI + SPC (incubation of plates at 32°C for 48 hours); PI + mPBC (incubation of plates at 21°C for 25 hours) (mPBC = modified psychrotrophic bacteria count on PCA); PI + CVT (One litre of PCA containing 1 ml of a 0.1% crystal violet solution followed by sterilization, cooling, and addition of 2, 3, 5- triphenyl tetrazolium chloride [TTC]) (Plates are incubated at 21°C for 48 hours.) (Marshall, 1992); PI + VRBA (incubation of plate at 32°C for 24 hours).
3. Bioluminescence.
4. Catalase detection.
5. Limulus amoebocyte lysate (LAL) assay—This procedure involves detection of endotoxins produced specifically by gram-negative bacteria (White, 1993).
6. Impedance microbiology.
7. Dye reduction (HR 1, HR2) (Randolph, Personal Communication, 1996).
8. Reflectance colorimetry (the LABSMART System (Gary H. Richardson, Logan, UT])—This is a tristimulus reflectance colorimeter that monitors dye pigment changes caused by microbial activity.

These methods reflect the most current information about the basics of shelf-life prediction techniques. However, no one procedure is ideally suited for every plant application.

21.3 MOSELEY KEEPING QUALITY TEST

The Moseley Keeping Quality Test consists of incubating the finished product in its original carton at 7°C for 5 to 7 days followed by doing the SPC, This test has been used for many years by dairy processors as a way of evaluating the "staying power" of their products. The big drawback is the length of time required for results, that is, 7 to 9 days before actual counts are obtained. As newer tests to predict shelf-life are developed, the tendency is for dairy processors using the Moseley Keeping Quality Test to correlate results of the new test with those of their regular test. This is not the way to evaluate a new test. The results of any test to predict shelf-life should be correlated with actual product shelf-

life not with the results of another test. Erroneous conclusions may be drawn. Thus, the best testing protocol is a preliminary incubation of the product so any psychrotrophs present can be enumerated rapidly. A multiple set of time and temperature combinations have been evaluated but the one set of conditions that seems to optimize outgrowth and enumeration of the psychrotrophs is incubation for 18 hours at 21°C. Therefore, the preliminary incubation (PI) mentioned in the remainder of this chapter represents 18 hours at 21°C.

21.4 PI + IDT TEST

Bishop and White (1985) used PI + impedance detection time (IDT) to successfully predict the shelf-life of fluid milk. For fluid milk products, the PI + IDT yielded the highest correlation (r = 0.94) between test result and actual product shelf-life at 7°C. By comparison, the correlation obtained for the Moseley Keeping Quality Test was r = 0.75. Because of the 7 to 9 days required before results are available from the Mosely test and because fluid milk products have a shelf-life of approximately 14 to 21 days at 7°C, there is no question which test would be of more value to the processor. Any of the tests discussed that can give results within 72 hours are of more value not only in predicting shelf-life but also in controlling the sanitary operation of the plant. Fung (1994), in an excellent overview of rapid detection methods, described 10 attributes of an ideal rapid or automated microbiological assay system for food:

1. Accuracy—Especially sensitive for false-negative results
2. Speed—Accurate results within 4 hours
3. Cost—Designed for each application
4. Acceptability—Must be "official"
5. Simplicity—Ideally "dip-stick" technology
6. Training—Adequate for test or kit
7. Reagents and supplies—Stability, consistency, availability
8. Company reputation—Performance of product is critical
9. Technical service—Rapid and thorough
10. Space requirements—Should not take up a whole laboratory

Most of the tests discussed meet most of these criteria.

21.5 VTSLM METHOD

Another method is described by Bishop (1988) as the Virginia Tech Shelf-Life Method (VTSLM), which involves a preliminary incubation (21°C) followed by simple plating. He describes this method as being reliable, accurate, relatively rapid, economical, and familiar to laboratory personnel. He advocates aseptically transferring 10 ml of a pasteurized fluid milk product into a sterile test tube and incubating the tube and its contents at 21°C for 18 hours. The sample is then mixed well and diluted 1:1000 with the diluted sample being plated on PCA and incubated at 21°C for 25 to 48 hours. He indicated that this method provides an estimate of the growth potential of psychrotrophic bacteria that may be present in the sample. The time variation for the plate incubation indicates the difference between agar and 3M-Petrifilm methods. If PCA is used, add 50 ppm of a filter sterilized solution of 2, 3, 5 triphenyl tetrazolium chloride (TTC) to the melted and cooled (44°C–46°C) agar before

pouring plates. Only the red colonies should be counted. Counts can then be extrapolated to indicate estimated shelf-life. Shelf-life categorization by VTSLM (Bishop, 1988, 1993) follows:

| | Petrifilm/Agar | |
Count (cfu/plate)	Total Count (cfu/mL)	Estimated Shelf-Life (Days)
≤ 1	$\leq 1,000$	≥ 14
1-200	1,000-200,000	10-14
≥ 200	$\geq 200,000$	≤ 10

By continuing to do the test to predict shelf-life on a regular basis and reacting to the results, confidence can be instilled from quality assurance and production standpoints. Most spoilage of fluid milk-type products occurs from presence of pseudomonads and related gram-negative bacteria. The tests discussed tend to emphasize detection and enumeration of gram-negative rods.

21.6 INDIRECT ELISA TEST

Gutierrez et al. (1997) reported on generating monoclonal antibodies against live cells of Pseudomonas fluorescens, which were used in an indirect ELISA format to detect Pseudomonas spp. and related psychrotrophic bacteria in refrigerated milk. The researchers indicated that development of an ELISA technique using these specific antibodies would facilitate rapid screening of refrigerated milk for detection of high concentrations of bacterial cells. They reported a good correlation (r = 0.96) between the colony numbers of psychrotrophic bacteria from commercial milk samples maintained at 4°C by the SPC method and the ELISA technique. These authors stressed the advantages of the indirect ELISA technique as being its versatility, simplicity, and speed.

There is still somewhat of an art in predicting the shelf-life of dairy products. Because there is no one perfect test for all needs, processors must carefully select the one or two tests that best fit into their overall quality assurance program. The key points (White, 1991, 1996) regarding prediction of shelf-life are as follows:

1. Know the actual potential shelf-life of the products as measured at 7°C (45°C).
2. Select the test to predict shelf-life that best fits the total program.
3. Routinely do the tests and develop a history, categorizing the results.
4. Ensure top management commitment to define a course of action in case product failure is projected by the tests.

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22

Indigenous (Indian) Dairy Products

22.1 INTRODUCTION

Certain dairy products are typically of Indian origin and manufactured exclusively in India. These are called Indian Indigenous dairy products. A large portion of milk is utilised in these products, which are manufactured in homes, by halwais (sweet makers) and organised dairies also. Quantitatively, ghee is the major product, but microbiologists are least concerned as it does not involve microbial activity due to absence of moisture. Other products come in the category of intermediate moisture foods, and are prone to microbial changes. These are khoa (mawa) and khoa-based sweets, chhanna and channa-based sweets, paneer, shrikhand and others. Gross composition and principle of manufacture of some indigenous products is given in Table : 22.1.

Table 22.1 : Chemical composition and principle of
manufacture of indigenous dairy products

Product	Manufacturing principle	Mositure (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)	Sugar (%)
Khoa	Open-pan dehydration to a semisolid consistancy	19.2	37.1	17.8	22.1	3.6	—
Burfi	Sugar addition in Khoa	8.8	8.9	15.2	8.3	2.3	52.0
Peda	Sugar addition in Khoa	8.8	9.2	9.1	9.4	2.0	55.0

contd. ...

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Product	Manufacturing principle	Mositure (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)	Sugar (%)
Kheer/ Basundi	Partial dehydration and addition of sugar and rice	67.0	7.8	6.3	8.4	1.4	8.9
Chhanna	Acid coagulation and draining	51.6	29.6	14.4	2.3	2.0	—
Rasogolla	Water fried chhanna dipped in sugar syrup	55.0	5.0	5.0	—	—	45.0
Paneer	Acid coagulation and draining	52.0	25.0	14.0	2.2	2.1	—
Rabri	Partial dehydration with sugar	30.0	20.0	10.0	17.0	3.0	20.0
Dahi	Fermentation	86.0	6.0	3.0	5.0	0.7	—
Chakka (Masko)	Fermentation and draining	59.6	22.4	10.3	4.4	1.0	—
Shrikhand	Blending chakka with sugar	35.0	6.0	5.0	3.0	—	50.0

22.2 KHOA AND KHOA BASED PRODUCTS

Khoa is made by continuous heating and stirring the milk, which destroys all the microorganisms. However, if the contamination takes place at the later stage, some organisms may survive, as with high solids, the heat resistance of the organism also increases. Some organisms, like Staphylococci, may be injured during the process and recover during storage. Generally, khoa, when made, is almost sterile, but gets heavily loaded with different organisms from humans, environment, utensils and packaging materials just after production. Many of the organisms are able to grow in khoa and bring about spoilage within 2 to 3 days only.

In khoa based sweets like peda or burfi, sugar is added and further heat treatment is done, which destroys most of the organisms. Sugar reduces the water activity, which restricts the microbial growth. Hence, peda, burfi and other sweets have higher shelf-life than khoa.

With respect to number and types of organisms in these products, vide variation occurs. Total count may vary from 10^4 to 10^9 per gram depending upon period and temperature of storage. In khoa and burfi the predominating organisms are Bacillus sp., Micrococci and Staphylococci. Peda is manually moulded, hence it gets more chances of staphylococcai contamination. The other bacteria belong to Coliforms, Streptococci, Lactobacilli, etc. Yeasts and molds are major spoilage organisms which come through air. Among the yeasts, Saccharomyces and among the molds Penicillium predominate in khoa.

Other major species are *Candida, Rhodotorula, Aspergillus, Geotrichum, Mucor* and *Syncephalostrum*.

The shelf-life of khoa can be improved by several measures as shown below:

(i)   Greater hygienic care,

(ii)   Steaming and packaging at high temperature,

(iii)   UV or gamma-irradiation,

(iv)   Controlling water activity by adding solutes like sugar,

(v)   Effective packaging, by use of good quality sanitized packs or gas or vacuum packing.

(vi)   Storage at low temperature and low humidity.

(vii)   Use of chemical preservatives like nisin, sorbates, etc.

Khoa has been implicated in many food poisoning cases. The major causative agent is *Staph. aureus*. Khoa is said to be an ideal medium for this organism as it is rich in nutrients, has low water activity (at which other organisms are inhibited but S. *aureus* can grow easily) and supplies growth factors like glycine, glutamic acid, proline and lactose-casein interaction products. The population of *Staph. aureus* can rise from initial 10^3 to 10^8 in 3-4 h at 37°C in khoa. In certain cases, the food poisoning has occurred even though no live organisms could be detected. This could be attributed to high level of their activity in milk, so that toxin is produced in milk itself. When khoa is made, organisms may die, but the heat resistant toxin remains active and causes food poisoning. The other pathogen which can grow and produce enterotoxin is *B. cereus*. However, no outbreaks of food poisoning due to this organism are reported. The growth of *E. coli, Shigella* and *Vibrio* can also pose a potential health hazard, if khoa is contaminated.

The most common defect is khoa in appearance of mold growth. Souring and off flavours can be produced by acid producers and coliforms.

22.3 BASUNDI AND KHEER

These are concentrated milks, added with sugar. Rice is additional component in Kheer. Sufficient information is not available about their microbiological quality, but may contain a variety of post-production contaminants, which can grow subsequently. These products are generally consumed immediately, but if stored and low temperature is not maintained, they can cause microbial hazards.

22.4 CHHANNA AND PANEER

These are heat and acid coagulated products, which are obtained after draining whey. High heat treatment involved in their manufacture, kills most of microorganisms initially present in milk. However, subsequent to heating they can get contaminated from coagulant solution, air, cloth, utensils, humans, water and packaging materials. These products are not able to restrict the growth of bacteria, yeasts or molds and hence, depending upon the age, contains variable numbers of acid producers, proteolytic, lipolytic, chromogenic and spore forming bacteria, yeast and molds. However, the activity of these microbes in chhanna and paneer depends upon the moisture content, pH, temperature of storage, packaging materials and handling practices.

Microbiological spoilage of these products occur within 24 to 72 h. Souring can be caused by lactic and non-latic acid producers, while lipolytic and proteolytic changes can be brought about by spore formers, Micrococci and Gram negative rods. Growth of molds and other chromogenic bacteria like Micrococci produces surface discoloration.

Paneer is used in culinary preparation, but chhanna is utilised for preparation of several sweets like rasogolla, sandesh etc.

22.5 CHAKKA AND SHRIKHAND

These are fermented milk products obtained from dahi. When a part of whey is drained from dahi, it gives a semi-solid mass, which is called chakka. It has about 60% moisture and 2% lactic acid. It is simply an intermediately product used in preparation of shrikhand. Shrikhand is prepared by blending chakka with sugar, flavour, cream, etc. and may also be added with fruits, and nuts. This is a very popular sweet dish in Western India. It contains 35-45% moisture, 40-45% sugar and have about 1% lactic acidity.

The microflora of shrikhand consists of lactic acid bacteria from starter. However, most of the lactic streptococci die out due to high acidity but Lactobacilli can dominate. The contamination from air, humans, utensils, sugar and other additives is very significant. The microbial growth during storage is not much problematic, because conditions in shrikhand are not favourable due to, i) high acid content, (ii) low pH, (iii) high osmotic pressure, (iv) low storage temperature and (v) low water activity. Chakka is more prone to spoilage than shrikhand because of high moisture and absence of added sugar in it.

Shrikhand, stored at room temperature sours due to continued activity of starters, mainly Lactobacilli. Coliforms, Bacilli and yeasts and molds can cause off flavours in shrikhand if conditions are conducive. Molds being osmnotolerant and aciduric, can grow on shrikhand and spoil it. Among measures to delay microbial spoilage and increase shelf-life, apart from good hygienic practices, 'thermization' is also suggested. This is the heat treatment of about 50-60°C to the finished product. This kills the remaining starter bacteria and other contaminants including molds. Popular Amul Shrikhand is a thermised product.

22.6 KULFI

Kulfi is an ice-cream like product made from sweetened concentrated milk added with 'malai' (cream), crushed nuts and flavour. It is filled in cone or moulds and frozen in mud pots or other containers using ice and salt. Unlike ice-cream, air is not incorporated in the product. The product is likely to contain various organisms coming from raw materials, utensils, air water, dust and humans. A survey has indicated that Staphylococci and Coliforms dominate in kulfi, while total count may vary from 10^5 to 10^7 per gram.

22.7 MICROBIOLOGICAL TESTS AND BIS STANDARDS FOR INDIGENOUS DAIRY PRODUCTS

Routine examination of indigenous dairy products can be done by total plate count, coliforms and yeast and mold counts. However, the products can also be analysed for specific group of bacteria like, acid producers, proteolytic, lipolytic, Chromogenic, etc. Tests

for pathogens like *Staph. aureus, Salmonella* and *B. cereus* are also recommended. Standards prescribed by BIS are given in Table : 22.2.

Table 22.2 : BIS prescribed microbiological standards for indigenous dairy products

	Product	Standard Plate count max. (cfu/g)	Coliform count, max. (cfu/g)	Yeast & Mold count, max. (cfu/g)
1.	Khoa	NS	90	50
2.	Burfi	30,000	NS	10
3.	Paneer	5,00,000	100	250
4.	Kulfi	2,50,000	100	NS
5.	Chakka	NS	10	20
6.	Shrikhand	NS	10	50
7.	Canned Rasogolla	500	Nil	NS
8.	Gulabjamum	3,000	50	50
9.	Sugar syrup for gulabjamun	5,000	50	50

NS - Not specified.

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23

MICROBIAL CONTROL BY
NEW NONTHERMAL METHODS

23.1 INTRODUCTION

Many of the conventional food preservation methods used at present and discussed here have several disadvantages. High heat treatment given to foods for safety and long shelf life results in loss of heat-sensitive nutrients (e.g., thiamine, riboflavin, folic acid, and vitamin C), denatures proteins and causes changes in texture, colour, and flavour, and induces formation of new compounds through covalent bondings (e.g., lysinoalanine). Low heat processing, such as pasteurization, minimizes the disadvantages of high heat processing of foods, but the foods have limited shelf-life even at refrigerated storage. Drying and freezing also reduce the nutritional and acceptance qualities of food, especially when stored for a long time. Irradiated foods have not been well accepted by consumers. Many of the chemical preservatives used are of nonfood origin and have limited efficiency.

Since the 1980s health-conscious consumers, especially in developed countries, have been concerned about the possible adverse effects that "harshly produced" and "harshly preserved" foods might have on their health and on the health of future generations. There is a concern that the cumulative effects of different types of food preservatives on the human body during one's life-time is not properly understood. The revelation about the harmful effect of some of the additives that were once allowed to be incorporated in foods has shattered consumer confidence. The philosophy of consumers has changed from, "How long will I live?" to "How well will I live?", which in turn has shifted the desire of these consumers to nutritious, natural, and minimally processed foods that have not been subjected to "harsh processing" or "harsh preservation" techniques. Due to changes in socio-economic patterns and lifestyles, many consumers are also interested in foods that have a long shelf-life and take very little time to prepare.

The suitability of several nonthermal processing and preservation methods are being studied to produce such foods. Some of these include high electric field pulses, oscillating magnetic field pulses, intense light pulses, and ultrahigh hydrostatic pressure. The principal advantages and disadvantages and the current status of these four methods are briefly described here. Major emphasis is given to their antimicrobial properties.

23.2 FOOD PRESERVATION BY HIGH ELECTRIC FIELD PULSES

The antimicrobial effect of high electric field pulses is not due to the electric heat or electrolytic products, but rather to the ability to cause damage to the cell membrane. When microbial cells, in a suspension, are exposed to pulses of high-voltage electric fields, a potential difference occurs between the outside and inside of the membrane. When the external electric field strength is moderately higher, so that transmembrane potential does not exceed the critical value by I V, pore formation occurs in the membrane, but the process is reversible (this principle is used in electroporation of cells to introduce foreign DNA). However, if a much higher external electric field strength is applied so that membrane potential exceeds the critical value, the pore formation becomes irreversible, causing the destruction of membrane functions and cell death. For destruction of microbial cells, an electric field strength of about 15 to 25 kV/cm for 2 to 20μs is necessary. Destruction of bacterial and fungal spores requires a higher voltage and a longer period of time.

The lethal effect of pulsed electric field against microorganisms has generated interest to use this for nonthermal pasteurization and commercial sterilization of foods. During the process, the temperature of the suspension increases very little. However, to obtain greater microbial destruction, the temperature of the suspension can be increased to 60°C or higher. Also, by increasing the number of pulses, greater microbial destruction can be achieved.

A process designated as "**Elsterile**" was developed in Germany for the microbial destruction by pulsed electric fields in liquid food. The liquid food, in a treatment chamber that has two carbon electrodes, is subjected to high-voltage electric pulses. A four log reduction was obtained for *Lactobacillus brevis* in milk by treatment with 20 pulses at 20 kV/cm for 20 μs. A similar reduction was also observed by treating *Saccharomyces cerevisiae* in orange juice with five pulses of 20-μs duration at 4.7 kV/cm. The increased reduction of the yeast cells, as compared to bacterial cells, was thought to be due to the low pH of orange juice and the larger cell size of yeasts.

In the United States a method was developed to preserve fluid foods such as dairy products, fruit juices, and liquid egg products. In this patented method, a food is subjected to high-voltage electric field pulses in a chamber fitted with two electrodes, at 12 to 25 kV/cm for 1 to 100 μs. In addition, the products are subjected to pasteurization followed by rapid cooling. The shelf-life of these products is much longer than conventionally pasteurized products.

These limited studies have shown that microorganisms in a liquid can be killed nonthermally by exposing them to high electric field pulses for a very short time. However, the method does not inactivate bacterial and fungal spores. Due to their nonthermal nature, it does not affect the texture, flavour, or colour of the products.

23.3 OSCILLATING MAGNETIC FIELD PULSES

Exposure of microorganisms to high-intensity oscillating magnetic fields tends to energize cell macromolecules (e.g., DNA and proteins) to the extent of breaking covalent bonds and rendering them metabolically inactive. For antimicrobial effect, a magnetic field intensity should be between 5 to 50 tesla (unit of magnetic field intensity) at a frequency of 5 to 500 kHz exposed for a total time of 25 μs to a few milliseconds. Limited studies have shown that such a treatment can reduce microbial population by 2 logs. This is a nonthermal process inasmuch as the temperature increases very little during the treatment. The antimicrobial efficiency at a certain magnetic field is dependent upon the total time of exposure and not on the number of pulses. Orange juice, milk, and yogurt were tested for antimicrobial effectiveness and acceptance quality following treatment with oscillating magnetic fields. In one study, the foods were sealed in plastic bags and subjected to 1 to 100 pulses in an oscillating magnetic field with a frequency of 5 to 500 kHz at 0 to 50^0C for a total exposure time of 25 μs to 10 ms. The temperature of the foods increased by 2 to 5°C and there were no changes in visual or organoleptic properties. Microorganisms were reduced by 3 to 4 logs. This method is safe and can be used for long storage of food along with other suitable methods (such as low heat).

23.4 INTENSE LIGHT PULSES

Energy released in short high-intensity pulses of light is known to kill microorganisms and deactivate enzymes. The light pulses are used for a fraction of a second and have a wavelength spectrum between 170 and 2600 nm. The wavelength spectrum used depends upon the type of food materials. For treating packaging materials, the wavelengths in the UV range are used; but for UV-sensitive food materials, wavelengths beyond UV range are used. The treatment does not increase the temperature of a food. The method can be used in addition to sterilizing packaging materials; for the surface sterilization of beef, pork, and poultry carcasses, fresh fish, vegetables, fruits, bakery goods, and solid dairy products in transparent packages; and bulk sterilization of transparent liquids devoid of solid particles. Test results revealed that bread slices treated through packaging materials maintained a fresh appearance for more than 2 weeks, while untreated controls became moldy. Fresh uncut tomatoes exposed to pulsed light remained acceptable at refrigerated temperature for over 30 d.

The pulsed light process units contain a series of hooded lamps that can be installed on-line for continuous on-site operation. Like other nonthermal processes, light pulses do not increase the temperature or adversely affect the texture, flavour, colour, or nutrient content of a food.

23.5 ULTRAHIGH HYDROSTATIC PRESSURE (UHP)

Microbial cells, when exposed to high hydrostatic pressure inside a pressure vessel containing water, die rapidly, especially at 14,500 psi and above (14.5 psi = 1 Bar = 1 Atm = 1 kg/cm^2 = 750 torr = 100 kilo Pascal or 0.1 mPa). Microbial death has been attributed to

the damage and loss of activity of the cytoplasmic membrane. In addition, damage to the cell wall (or outer membrane), deactivation of intracellular enzymes, and the inability of amino-acyl t-RNA to bind to ribosomes were observed. The death curve of a microbial species shows characteristics of thermal destruction curve; i.e., initially there is a rapid rate of reduction of viability, followed by a slow rate and then tailing. In general, the death rate is higher: for young cells as compared to stationary phase cells; for rods as compared to cocci; in water suspension as compared to in a broth; at a higher pressure as compared to a lower pressure; and with longer time as compared to a shorter time. Surviving cells show repairable injury in the wall and membrane. Bacterial spores are killed at very high pressures (generally >100,000 psi). However, spores of some *Bacillus* spp. show more death in the 15,000- to 45,000-psi range than at higher pressure. This is thought to be due to induction of spore germination at lower pressure, followed by outgrowth of cells which, like vegetative cells, are then killed by the pressure. Depending upon the different factors mentioned above, as much as 5 to 6 logs or more reduction of bacterial cells can be achieved at pressures ≤100,000 psi.

Application of ultrahigh hydrostatic pressure (between 15,000 and ≤ 150,000 psi) has been advocated to reduce microbial counts for the preservation of foods. In the process, the food in a bag or a container is suspended in a liquid (generally water mixed with a little oil) in a pressure chamber. After closing the chamber, the pressure is raised by pumping more liquid into the closed chamber. The pressure is immediately and uniformly transmitted into the food (and microorganisms present in it). At high pressure, water shrinks relatively little (as compared to gas); specifically, about 4% at 15,000 psi, 11% at 60,000 psi, and 15% at 87,000 psi. Also, the temperature remains essentially unchanged. Since the pressure is uniform around a food exposed to hydrostatic pressure, it does not undergo substantial changes in acceptance qualities, especially in a low pressure range (≤60,000 psi).

Among food components, proteins are denatured by high hydrostatic pressure due to the destruction (and reformation) of hydrogen bonds, ionic bonds, and hydrophobic bonds, which only affect the tertiary structures; covalent bonds are not affected. The changes in the original tertiary structure from breaking and reformation can change coagulation or gelation characteristics of some foods, giving them a unique and novel texture. However, high-pressure treatment does not reduce the flavour or nutrient content of a food.

Applications of UHP for the processing and preservation of different types of foods are being studied in many countries. Studies as early as 1914 revealed that the microbial level in milk was reduced by about 6 logs when exposed to 100,000 psi for 10 min at room temperature, and meat exposed to 78,000 psi for 1 h at 52^0C in a bag did not show any deterioration during the 3 months of the study periods. Also, fruit juices and fruits subjected to hydrostatic pressure at ≤100,000 psi had a long shelf life. In recent years, the UHP process has been effectively applied to many products to reduce microbial load and increase shelf life. These include fresh fruit juices, fresh jellies and jams, whipped frozen strawberries, apple pieces in syrup, crushed fresh tomatoes, fresh vegetable juices, raw fish, meat, ground meat, meat products, fruit cocktail, spaghetti in sauce, rice in sauce, and vegetables. These products have more natural colour, more flavour, and an extended shelf-life. It has been successfully applied to the commercial production of fresh fruit juices, jams and jellies, and coffee and tea. The antimicrobial effect of high hydrostatic pressure has also been studied

as it relates to the inhibition of undesirable growth of lactic acid bacteria in yogurt and possible accelerated ripening of cheeses. Pressure treatment is also being tested to quickly kill insects and parasites in foods.

As indicated before, ultrahigh hydrostatic pressure, in addition to antimicrobial action, induces some textural and colour changes in some foods, especially proteinaceous foods. Some of these changes are being used to develop innovative and better products. When eggs are subjected to high pressure ($\sim$ 90,000 psi), the white and yolk coagulate to form a gel. The gel is more lustrous than heat-induced gels, has a natural taste (without cooked flavour), and a soft, elastic (as adhesive) texture that does not break easily. The nutrient content remains unchanged. This gelling property can be used to produce egg-containing products with better texture, flavour, and nutrients. Fish paste, meat paste, and soy protein paste also form gels following high hydrostatic treatment that have desirable rheological and organoleptic properties than heat-induced gels. The gels are also smooth and glossy. This method is being used to make different types of surimi.

Pressure treatment of proteins and starches can make them susceptible to the hydrolyzing action of proteinases and α-amylase. In addition, starches also undergo gelatinization. Thus pressure treatment can also be used to enhance enzyme digestion of proteins and starches where required.

Beef muscle is tenderized at low hydrostatic pressure (15,000 psi for 4 min at 35°C prerigor or 22,000 psi for 1 h at 60°C postrigor). The pH also drops by 0.3 at 44,000 psi. Tenderization is probably brought about by the proteolytic action of cathepsins released from the lysosomes in the cytoplasm. Tenderization in beef by pressure seems to occur by a different mechanism than that which occurs during conditioning. The increase in flavour in the pressurized meat may be due to the increase in flavour compounds (amino acids or peptides). Tenderization of lower grade beef can be achieved by high-pressure treatment. However, myoglobin in fresh meat may lose the bright red colour above 44,000 psi. Beef treated at 58,000 psi for 10 min may look like ham without any change in beef flavour; when lightly baked, it tastes like rare steak without loss of liquid.

In addition, high hydrostatic pressure is being studied for other innovative food processing technologies. At high pressure, the freezing point of water decreases: namely —10°C at 20,000 psi and —20°C at 29,000 psi. For a long shelf life, foods can be stored at low temperature (—10°C or below) by moderately low hydrostatic pressure without freezing. Moderately low pressure was observed to cause rapid thawing of frozen foods without causing any loss of liquid or other changes.

At present, high hydrostatic pressure in food can be used for hatch processing. Technology for this process is rapidly progressing and equipment that will achieve pressures >150,000 psi is being produced. Pressure vessels with a capacity of several thousand litres are now in operation. In addition, methods for semi-continuous operation are being developed. As the process is instantaneous and may not take a long time, there is suggestion that a unit can be recycled rapidly. However, that might reduce time service life of a unit due to metal fatigue.

The influence of other parameters are being studied along with the pressure at a lower range ($\leq$60,000 psi). They include several chemical antimicrobial agents. Units are

now available in which the temperature of the liquid in the chamber can be raised to 90°C or higher. In addition, biopreservatives, such as bacteriocins of lactic acid bacteria, lysozyme, and chitosan (a polycationic derivative of chitosan), can be added to enhance the antimicrobial effect of hydrostatic pressure treatment.

Ultrahigh hydrostatic pressure technology has great potential in the preservation and processing of foods. From the progress made since the mid-1980s and the interest it has generated in many countries among food processors, government agencies, and researchers, ultrahigh hydrostatic pressure treatment may be one of the most important nonthemmal processing and preservation methods of food in the next century.

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DAIRY WASTES AND TREATMENT

24.1 INTRODUCTION

With increase in demand for milk and milk products, many dairies of different sizes have come up in different places. These dairies collect the milk from the producers, and then either simply bottle it for marketing, or produce different milk foods according to their capacities. Large quantity of waste water originates due to their different operations. The organic substances in the wastes comes either in the form in which they were present in milk, or in a degraded form due to their processing's. As such, the dairy wastes, though biodegradable, are very strong in nature.

24.2 SOURCES OF WASTE

The liquid wastes from a large dairy originate from the following sections or plants: receiving station, bottling plant, cheese plant, butter plant, casein plant, condensed milk plant, dried milk plant, and ice cream plant. Waste also comes from water softening plant and from bottle and can washing plants.

At the receiving station the milk is received from the farms and after inspection the same is emptied into large containers for transport to bottling or other processing plants. The empty cans are rinsed, washed, sterilized and are returned to the farmers.

At the bottling plant, the raw milk delivered by the receiving station is stored. The processing includes cooling, clarification, filtration, pasteurization, and bottling.

In the above two sections, the liquid wastes originate out of rinse and washings of bottles, cans and equipments, and thus contain milk drippings and chemicals used for cleaning containers and equipments.

In a cheese plant, the milk (whole milk or skimmed milk) is pasteurized and cooled and placed in a vat, where a starter (lactic acid producing bacterial culture) and rennet are added. This separates the casein of the milk in the form of curd. The whey is then withdrawn

and the curd compressed to allow excess whey to drain out. Other ingredients are now added, and the cheese blocks are cut and packaged for sale. Waste water from this plant include mainly the discarded whey and the wash water used for cleaning vats, equipments, floors etc.

In the creamery process, the whole milk is preheated to about 30°C to separate the cream from the milk. In a butter plant the cream is pasteurized and may be ripened with a selected acid and a bacterial culture. This is then churned at a temperature of about 7-10°C to produce butter granules. At a proper time the butter milk is drained out of the churn and the butter is washed and after standardisation, packaged for sale. Butter milk and wash waters used to clean the churns, and small quantity of butter comes out as the waste from the butter plants.

The skimmed milk may now be sent for bottling for human consumptions, or for further processing in the dairy for other products like non fat milk powders. Milk powders are produced by evaporation followed by drying by either roller process or spray process. The dry milk plant wastes consists chiefly of wash waters used to clean containers and equipments.

The soured or spoiled milk, and sometimes the skimmed milks are processed to produce caseins used for preparation of some plastics. The process involves the coagulation and precipitation of the casein by the addition of some mineral acids. The waste from this section includes whey, washings and the chemicals used for precipitation.

Very large dairies also produce condensed milk and Ice-creams.

In addition to the wastes from all the above milk processing units, some amount of uncontaminated cooling water comes as wastes; these are very often re-circulated.

The dairy wastes are very often discharged intermittently; the nature and composition of waste also depend on the types of products produced, and the size of the plants. The Table 24.1 gives the characteristics of the waste (composite) of a typical Indian Dairy, handling about 300000 to 400000 litres of milk in a day.

Table 2.4.1 : Composition of the waste water of a typical dairy

Item	Value
pH	7.2
Alkalinity	600 mg/l as $CaCO_3$
Total dissolved solid	1060 mg/l
Suspended solid	760 mg/l
BOD	1240 mg/l
COD	84 mg/l
Total nitrogen	84 mg/l
Phosphorous	11.7 mg/l
Oil and grease	290 mg/l
Chloride	105 mg/l

24.3 EFFECTS OF THE WASTES ON THE RECEIVING STREAMS/SEWERS

As observed from the above table the waste is basically organic in nature. This is also slightly alkaline when fresh. When these wastes are allowed to go into the stream

without any treatment, a rapid depletion of the dissolved oxygen content of the stream occurs, along with growth of sewage fungi covering the entire bottom of the stream and the submerged parts of the hydraulic structures within it. The waste is said to carry, occasionally, the bacteria responsible for tuberculosis. Though alkaline in fresh condition, the milk waste becomes acidic due to the decomposition of Lactose into lactic acid under anaerobic condition, particularly after complete oxygen depletion of the stream. The resulting condition precipitates casein from the waste, which decompose further into a highly odorous black sludge. At certain dilutions the dairy waste is found to be toxic to fishes also.

As the dairies are usually situated in rural areas or in small towns, the question of discharging the dairy waste into the sewers does not arise. In large cities, combined treatment of domestic sewage and dairy waste may be considered if the latter constitutes only 10% in volume of the former. In that case the dairy waste should be discharged in a fresh condition, as a putrefied waste may cause corrosion of the sewers.

24.4 TREATMENT OF THE DAIRY WASTES

As evident from the low COD: BOD ratio, the dairy wastes can be treated efficiently by biological processes. Moreover, these wastes contains sufficient nutrients for bacterial growth. But for economical reasons, attempt should be made to reduce volume and strength of the waste. This may be accomplished by (i) the prevention of spills, leakages and dropping of milks from cans, (ii) by reducing the amount of water for washes, (iii) by segregating the uncontaminated cooling water and recycling the same, and (iv) by utilising the butter milk and whey for the production of dairy byproducts of good market value.

Due to the intermittent nature of the waste discharge, it is desirable to provide Equalization tank, with or without aeration, before the same is sent for biological treatment. A provision of grease trap is also necessary as a pre-treatment to remove fat and other greasy substances from the waste. An aeration for a day not only prevents the formation of lactic acid, but also reduces the BOD by about 50%.

Both high rate trickling filters, and activated sludge plants can be employed very effectively for a complete treatment of the dairy waste. But these conventional methods involve much maintenance, skilled personnel, and special type of equipments. On the other hand the low cost treatment methods like oxidation ditch, Aerated Lagoon, Waste Stabilisation Pond etc. can be employed with simpler type of equipments and less maintenance.

Oxidation ditches in India may be designed with a low organic loading (about 0.2 kg/kg of MLSS), high biological mass concentration (in the order of 4000 mg/l), and extended period of aeration (in the order of 1.5 days), for BOD reduction of about 95 to 98%.

In a waste stabilisation pond, a BOO reduction of 52 to 74% could be achieved after 12 days of retention and an organic loading of 550 to 585 kg/hectare/day.

BOD reduction of about 90% may be obtained with a retention time of 7 days and a depth in the order of 3 m, in an anaerobic lagoon. An organic loading in the order of 0.48 kg/m^3/day is suggested for the above.

Use of dairy waste for Irrigation after primary treatment in an Aerated lagoon may also be good answer for the disposal of Dairy waste.

24.5 AN OUTLINE DESIGN FOR 2,00,000 L/DAY-CAPACITY EFFLUENT TREATMENT PLANT FOR MILK DAIRY

The main aim of effluent treatment plant in milk dairy is to treat the effluent to avoid pollution of water, environment and bad effects on public health and to improve sanitation of milk dairy plant.

The requirements and treatment procedure for an effluent plant having 2,00,000 litres/day capacity in milk dairy are outlined as follows:

(1) Equalization Tank (2 Nos.)—The waste water entering into the tanks is aerated by means of HDPE aeration grid provided at the bottom of the tank to mix the waste. Here lime slurry is added to it. Then this waste is pumped to the flash mixer tanks.

Dimensions—9 m × 9 m × 2 m R.C.C. construction

(2) Flash Mixer Tank—This is provided with flash mixer mechanism to mix the waste water with alum solution. This tank receives solution as alum form the alum solution tank.

The content of this tank overflows to the clariflocculator.

Dimensions—0.8 m × 0.8 m × 0.8 m R.C.C construction

(3) Clariflocculator—Waste from the flash mixer tank is received in the central flocculation zone. Here lime slurry and alum solution is mixed slowly by flocculation paddles and due to this suspended matter coagulates to form flocs which settle down. The mechanism has a rotating skimmer, which skims out the floating matter which is collected in tray fitted, on the top and is drawn out of the tank through a pipe connection to the tray.

The mechanism also has a rotating scraper at the bottom outlet of the tank and these solids in the form of sludge are drawn out from the outlet and are dried over sludge drying beds.

And partly treated waste water overflows out to the aeration tank.

(4) Aeration Tanks (2 Nos.)—This is a tank of R.C.C. construction and of dimensions 12 m × 12 m × 3 m. It is provided with salec surface aerator of capacity 30 HP. The tank receives partly treated waste water from the clariflocculator and overflows to the clarifier. The aerator provides required amount of oxygen for the process.

(5) Clarifier—This received overflows of the aeration tanks in the central well. This is circular R.C.C. tank of dimension 8 m × 2.5 m.

In this tank, the suspended solids settles down and clear supernatant liquid overflows out as treated waste. The settled solids are removed out by the rotating scrapper of the mechanism and are pumped back to the aeration tank in the form of sludge.

(6) Sludge Drying Beds—This tank receives the sludge from clariflocculator tank and the clarifier tank. The solids from the sludges are dried on the top which are removed periodically and the liquid seeps through the media and flows through the bottom channel. Out of the tank media is consisting of gravels and sand. There are four tanks each of dimension 15 m × 15 m and bottom of the tank is slopped and fitted with the filtering media.

(7) Raw Effluent Pumps (2 Nos.)—These are provided to pump the raw waste water from equalization tank to the flash mixer tank.

(8) Alum/Lime Slurry Preparation Tanks (2 Nos.)—These are tanks of R.C.C. construction each of dimension 1 m × 1 m × 1 m, provided with paddle agitator to prepare slurry of alum and lime respectively.

(9) Blowers (2 Nos.)—These are for air injection to the equalization tanks through the aeration grids. These are in the pump house, having a capacity of 140 m^3/hour.

(10) Return Sludge Pumps (2 Nos.)—These are installed near clarifier, which pumps the bottom sludge of the clarifier to the aeration tanks. Capacity of the pump is 30 m^3/hour.

(11) Disposal—Disposal of effluent after giving above treatment (after lowering down its suspended solids content and B.O.D.), for the irrigation purpose of the land.

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Appendix A

TAXONOMIC CHARACTERISTICS OF STARTER CULTURES

1. *LACTOCOCCUS*

Lactococci (formerly group N streptococci) are the major mesophilic microorganisms used for acid production in dairy fermentations. Although five species are recognized, only one, *Lactococcus lactis*, is of significance in dairy fermentations. *Lc. lactis* cells are cocci that usually occur in chains, although single and paired cells are also found. They are homofermentative; when grown in milk, more than 95% of their end product is lactic acid (of the L isomer). Lactococci grow at 10°C but not at 45°C. They are weakly proteolytic and can use milk proteins. There are two subspecies, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. Differential characteristics for these subspecies are presented in Table : 1. *L lactis* subsp. *locus* is more heat and salt tolerant than *Lc. lactis* subsp. *cremoris*. A variant of *Lc. lactis (Lc. lactis* subsp. *locus* var. *diacetylactis)* converts citrate to diacetyl, carbon dioxide, and other compounds. Some lactococci produce exopolysaccharide (Cerning, 1990). These variants are used to produce Scandinavian cultured milks having a ropy texture (viilli, taettamilk, and langmjolk), Another variant of *Lc. lactis* produces malty off-flavour caused by aldehyde production from amino acids (Morgan, 1976).

2. *STREPTOCOCCUS*

The only *Streptococcus* sp. useful in dairy fermentation is *Streptococcus thermophilus*. This microorganism is genetically similar to oral streptococci *(Streptococcus salivarius)* but can still be considered a separate species (Axelsson, 1993). *S. thermophilus* is differentiated from other streptococci (and lactococci) by its heat resistance, ability to grow at 52°C, and ability to ferment only a limited number of carbohydrates (Axelsson, 1993). Most dairy products subjected to high temperatures during fermentation (>40°C) are acidified by the combined growth of *S. thermophilus* and *Lactobacillus* spp. *S. thermophilus* has limited proteolytic ability.

Table 1 : Differentiation of Lactococci used in starter cultures

	Lactococcus lactis subsp.	
Characteristic	*Lactis*	*Cremoris*
Acid from		
Lactose	+	+
Galactose	+	+
Maltose	+	−
Ribose	+	−
Growth in 4% salt	+	−
Arginine hydrolysis	+	−

Source: Schleiter *et al.*, 1985.

3. *LEUCONOSTOC*

Leuconostoc spp. are distinguished from other lactic acid bacteria by being mesophilic heterofermentative cocci. They do not hydrolyze arginine and require various B vitamins for growth. *Leuconostoc* spp. used in the dairy industry produce diacetyl, carbon dioxide, and acetoin from citrate. Some also produce exopolysaccharide (dextran) from sucrose. Only two species of *Leuconostoc* are associated with dairy starter cultures, *Leuconostoc mesenteroides* subsp. *cremoris* (previously, *Leuconostoc citrovorum*) and *Leuconostoc lactis*. These are differentiated by their ability to ferment various carbohydrates. *Leuconostoc* spp. grow poorly in milk, probably because they are adapted to growth on vegetables and roots (Vedamuthu, 1994) and therefore lack sufficient proteolytic ability to grow in milk. *Leuc. mesenteroides* subsp. *cremoris* does not produce sufficient acidity in milk to coagulate it, but *Leuc. lactis* may (Thunell, 1995). In starter cultures, *Leuconostoc* spp. are combined with lactococci when production of diacetyl and carbon dioxide is desired in addition to acidification. When used in cultured milk starters, they convert excess acetaldehyde to diacetyl, thus reducing undesirable "green" flavour (Lindsay *et al.*, 1965). *Leuconostoc* spp. do not grow well in high-phosphate phage-inhibitory media (Vedamuthu, 1994).

4. *LACTOBACILLUS*

The *Lactobacillus* genus consists of a genetically and physiologically diverse group of rod-shaped lactic acid bacteria. The genus can be divided into three groups based on fermentation end products. Species in each of these groups can be found in dairy starter cultures, as listed in Table : 2. Homofermentative lactobacilli exclusively ferment hexose sugars to lactic acid by the Embden-Meyerhof pathway. They do not ferment pentose sugars or gluconate. These are the lactobacilli *(Lb. delbrueckii* subsp. *bulgaricus, Lb. delbrueckii* subsp. *lactis,* and *Lb. helveticus)* commonly found in starter cultures. They grow at higher temperatures (>45°C) than lactobacilli in the other groups and are thermoduric. Another member of this group, *Lactobacillus acidophilus* is not a starter culture organism, but is added to dairy foods for its nutritional benefits.

Facultatively heterofermentative lactobacilli ferment hexose sugars either only to lactic acid or to lactic acid, acetic acid, ethanol, and formic acid when under glucose

Table 2 : Characteristics of *Lactobacillus* spp. associated with dairy products

Species	Products	Growth at 15°C	Growth at 45°C	Lactic acid isomer	Mole %G+C	Fermentation of					
						Glu	Gal	Lac	Mal	Suc	Rib
Homofermentative											
L delbrueckii	Yogurt, kumiss, kefir,	−	+	D	49-51	+	−	+	−	−	−
subsp. *bulgaricus*	Italian, and Swiss cheeses										
subsp. *lactis*	Hard cheese	−	+	D	49-51	+	d[a]	+	+	+	−
L acidophilus	Acidophilus milk, laban	−	+	DL	34-37	+	+	+	+	+	−
L helveticus	Yogurt, Swiss cheese	−	+	DL	38-40	+	+	+	d	−	−
Facultatively heterofermentative											
L casei											
subsp. *casei*	Hard cheese	+	−	L	45-47	+	+	d	+	+	+
Obligately heterofermentative											
L. kefir	Kefir	+	−	DL	41-42	+	−	+	+	−	+

[a]Some strains are positive.

Abbreviations: Glu, glucose; Gal, galactose; Lac, lactose; Mal, maltose; Suc, sucrose; Rib, ribose.

Source : Cogan, 1996.

limitation. Pentose sugars are fermented to lactic and acetic acid via the phosphoketolase pathway. This group includes *Lactobacillus casei*, which is not usually found in starter cultures but is associated with beneficial secondary fermentation during cheese ripening.

Obligately heterofermentative lactobaeilhi ferment hexose sugars to lactic acid, acetic acid (or ethanol), and carbon dioxide using the phosphoketolase pathway. Pentose sugars are also fermented using this pathway. These lactobacilli can cause undesirable flavour and gas formation during ripening of cheese. One species, *Lactobacillus kefir*, is associated with kefir cultures.

Lactobacilli are the most acid tolerant of the lactic acid bacteria, preferring to initiate growth at acidic pH (5.5-6.2) and lowering the pH of milk to below 4.0. Lactobacilli are slow to grow in milk in pure culture. For this reason, they are generally used in combination with *S. thermophilus*.

5. PROPIONIBACTERIA

Propionibacterium spp. are non-spore-forming, pleomorphic, gram-positive rods that produce large amounts of propionic and acetic acid and carbon dioxide from sugars and lactic acid. They are anaerobic to aerotolerant mesophiles. They are not considered belonging to the lactic acid bacteria, but are closely related to coryneform bacteria in the Actinomycetaceae group. Four species of *Propionibacterium* are found in cheese (Table : 3), but *Propionibacterium freudenreichii* subsp. *fteudenreichii* and *P. freudenreichii* subsp. *shermanii* are most often used in cheese manufacture (Lyon and Glatz, 1995). Although *Propionibacterium* spp. are found in raw milk, they may be present in insufficient numbers to produce an adequate fermentation, so they are often added along with the lactic culture.

Table 3 : Differentiation of *Propionibacterium* spp. associated with dairy products

Characteristic	*P. freudenreichii*	*P. jensenii*	*P. thoenii*	*P. acidipropionici*
Acid from				
Sucrose	−	+	+	+
Maltose	−	+	+	+
Mannitol	−	+	−	+
Rhamnose	−	−	−	+
Nitrate reduction	−	−	−	+
β-hemolysis	−	−	+	−
Colony colour	Cream	Cream	Red-brown	Cream to orange-yellow

Source: Commins and Johnson, 1984.

Propionibacteria can use both inorganic and organic nitrogen sources, and their requirements for amino acids vary. Most strains require biotin. Cultures for cheese, manufacture are grown on complex media, including hydrolyzed protein and yeast extract with lactic acid as a carbon source (Glatz, 1992).

Propionibacteria grow on the lactic acid produced during cheese fermentation. The lactate is oxidized to pyruvate, which then is either converted to acetate and carbon dioxide or propionate. The carbon dioxide forms the large eyes found in Swiss and similar types of

cheese, and the other metabolic products including amino acids and fatty acids contribute to the flavour of these cheeses.

6. BREVIBACTERIUM

Brevibacteriuin cells are aerobic, gram-positive, pleomorphic rods that grow on the surface of surface-ripened cheese varieties. The species most often isolated from these cheeses is *Brevibacterium linens*. *B. linens* produces a yellow-orange carotenoid pigment that colours the surface of the cheese. Colour formation is enhanced by exposure to light. Older cultures are primarily coccoid, but slender rods are produced in exponential growth. *B. linens* does not use lactose or citrate but can grow on the lactate produced during cheese manufacture. It also grows best at neutral pH, so it does not grow well on the cheese surface until lactic acid is neutralized or metabolized by yeasts or micrococci. Surface-ripened cheeses are surface-salted and *B. linens*, like yeasts and micrococci, grows well at high salt concentrations. *B. linens* is highly proteolytic with ability to degrade whey proteins and casein (Fringa *et al.*, 1993; Holtz and Kunz, 1994). The ability of *B. linens* to degrade amino acids to ammonia and methionine to methanethiol is partially responsible for production of strong flavours and odours during surface ripening of cheese. Other volatile compounds produced by *B. linens* that contribute to the typical flavour of surface-ripened cheese include butyric acid, caproic acid, phenylmethanol, dimethyldisulphide, and dimethyltrisulphide (Jollivet *et al.*, 1992). *B. linens* grows well in media containing hydrolyzed protein, glucose, yeast extract, potassium phosphate, and magnesium sulphate (Haysahi *et al.*, 1990).

7. PENICILLIUM

Penicillium spp. are molds in the class Hyphomycetes in the division Deuteromycota. Molds in this class produce conidia directly on mycelium or on conidiophores. The conidiophores of *Penicillium* spp. arise erect from the hyphae and branch near the tip to produce a brush like ending (Beneke and Stevenson, 1987). Two groups of *Penicillium* spp. are used in cheese manufacture, the white mold *(Penicillium camemberti* Thorn, formerly two species, *Penicillium cuseicolum* and *Penicillium camemberti)*, which grows on the surface of Camembert, Brie, and similar varieties, and the blue mold *(Penicillium roqueforti*, formerly *P. roqueforti* var. *roqueforti)*, which grows in the interior of blue-veined cheeses such as Roquefort, Gorgonzola, and Stilton. *P. camemberti* is closely related to *Penicillium commune*, a common cheese contaminant that produces various toxins (Frisvad and Filtenborg, 1989), whereas *P. camemberti* produces only one mycotoxin, cyclopiazonic acid. *P. roqueforti* is closely related to *Penicillium carneum* (formerly *P roqueforti* var. *carneum)*, a producer of the mycotoxin patulin, and *Penicillium paneum* (formerly *P. roqueforti* var. *carneum)*, a producer of patulin and the mycotoxin botryodiploidin (Boysen *et al.*, 1996).

P. camemberti and *P. roqueforti* are lipolytic and proteolytic. Both produce methyl ketones and free fatty acids, but the much higher levels produced by *P. mqueforti* give blue cheeses their distinctive flavour and aroma (Kinsella and Hwang, 1976; Jollivet *et al.*, 1993). *P. camemberti* contributes to the flavour of Camembert and Brie cheeses by producing a complex mixture of compounds, the major ones being 2-heptanone, 2-heptanol, 8-nonen-2-one, 1-octen-3-ol, 2-nonanol, phenol, butanoic acid, and methyl cinnamate (Moines et al., 1975).

Appendix B

DIFFERENTIAL STAINING TECHNIQUES

Following two techniques are most common differential staining used in Bacteriology—

(I) Gram staining.

(II) Acid -fast staining.

(1) GRAM STAINING

(A) Introduction

Gram staining is one of the most important and widely used differential staining techniques in microbiology.

Christian Gram in 1883, while working on the etiology of respiratory disease in the municipal hospital in Berlin discovered this technique. Gram staining is a differential staining technique by which bacteria are classified as "Gram positive" or "Gram negative" depending upon whether they retain or lose the primary stain crystal violet; when subjected to treatment with a decolourizing agent such as alcohol. Bacteria are classified as "Gram positive" or "Gram negative" according to their response to the Gram staining procedure. Gram reaction is based on physico-chemical nature of cell wall of bacteria.

In this technique, the heat fixed bacterial smear is subjected to the following staining regents in the order listed: Crystal violet (**Primary stain**); Gram's Iodine (**Mordant**); Alcohol (**Decolourizer**) and safranine (**Counterstain** or **Secondary stain**).

Gram positive bacteria retain the crystal violet and appear deep violet in colour whereas Gram-negative bacteria lose the crystal violet on application of decolourizer and are counterstained by the safranine, hence appear red in colour.

(B) Principle/Mechanism/Theory

The difference between Gram - positive and Gram-negative bacteria has been shown to reside in the cell wall. Although the chemical composition of Gram-positive and Gram-negative walls, is now fairly well known, the reason gram-positive walls block the dye-extraction step is still unclear. The basic mechanism of Gram reaction is still not completely understood. Several theories have been proposed by various scientists to explain the

mechanism of Gram reaction. The most plausible and accepted hypothesis for this phenomenon is based on the difference in structure and composition of cell walls of Gram-positive and Gram-negative bacteria proposed by **M.R.J. Salton**. The cell wall of Gram-negative bacteria posses higher percentage of lipids (10-20%) as compared to that in the cell wall of Gram-positive bacteria (1-2%).

The primary stain [crystal violet -cv] forms a complex with the mordant Gram's Iodine (CV - I complex) in the cell. When bacterial cells are treated with decolourizing agent - alcohol, lipids from the cell wall of Gram-negative bacteria are extracted, this results in increased porosity or permeability property of the cell wall. Therefore CV-I complex escapes the cells and thus Gram-negative organisms are decolourized. In subsequent step, to render the colourless Gram-negative bacteria visible, counterstain - safranin is applied. These cells take up the colour of safranin, a basic dye. In contrast to this, the cell of Gram-positive bacteria because of their low lipid content, become dehydrated during treatment with alcohol. The cells shrinked in size, this in turn decreases pore size and permeability and the CV - I complex can not be extracted but it is retained in the cell. Therefore, Gram- positive cells appear purple violet in colour.

Another hypothesis is also based on permeability differences between Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, the CV-I complex is trapped in the cell wall following alcohol treatment which causes a dimuniation in the diameter of the pores in the cell-wall peptidoglycan. The cell walls of Gram-negative bacteria have a very much smaller amount of peptidoglycan (5-10 % of dryweight of cell wall) as compared to cell walls of Gram-positive bacteria (40-90 % of dry weight of cell wall). In Gram-negative bacteria, there appear to.be only one sheet of peptidoglycan; when, as in Gram positive bacteria there are as many as 40 sheets of peptidoglycan. The peptidoglycan in cell-wall of Gram-negative bacteria is less extensively cross linked and rather thin than that in cell wall of Gram-positive bacteria. The pores in the peptidoglycan of Gram negative bacteria remain sufficiently large even after alcohol treatment to allow the CV-I complex to be extracted.

These two hypotheses are not mutually exclusive and it is likely that both provide explanation of the mechanism of Gram reaction.

Gram reaction is of great **taxonomic importance** and is often the first step in the identification of an unknown procaryotic microorganism. For eucaryotic cells, this reaction is not significant since most of them are Gram-negative except eucaryotic microorganisms such as yeasts and few molds which are Gram-positive. In general Gram negativity is wide spreaded than Gram positivity in micorbial world.

(C) Procedure

(1)   Prepare a smear from the given bacterial culture on a clean sterile glass slide and allow it to air dry and fix it by heat treatment.

(2)   Cover the smear with Gention violet staining solution for 1 to 2 minutes.

(3)   Pour off the stain and wash the slide in running tap-water.

(4)   Cover the film with Gram's Iodine solution for 30-60 Seconds.

(5)   Rinse the slide in running tap water.

(6)   Apply 95 % ethanol or Acetone - alcohol mixture in a dropwise manner until excess violet colour ceases to come out and only faint violet colour remains in the solvent. During decolourization, the slide is held in inclined position.

(7)   Rinse the slide briefly in tap water.

(8)   Apply counterstain safranin and allow it to react for 3 to 4 minutes.

(9)   Pour off the stain and wash the slide in tap water, drain, blot and air dry.

(10) Examine the stained smear under oil immerson objective lens.

Result

Gram-positive bacteria are stained violet in colour while Gram-negative bacteria are stained pink in colour.

(D) Modifications of Technique

(1)   Method of Hucker and Conn and (2) Method of Preston and Morell are the examples of modifications of original Gram staining procedure.

(E) Importance of Gram staining

It is difficult to overemphasize how fundamental Gram stain classification has become important in bacteriology. The positive or negative staining reaction of a bacterium is still one of the first items mentioned in its description. Gram stains are also of considerable practical importance in medicine. As a general rule, drugs work optimally either on Gram-positive bacteria or Gram-negative bacteria; therefore, treatment of any infection is often based in part on a rapid determination of the Gram reaction of the disease-causing organism. Once that is known, then an appropriate drug can be administered even if complete identification of the organism has not been made.

Serious medical consequences can result when a laboratory error occurs in the Gram staining procedure. In one such instance, a patient suffering form an unknown disease had a high fever and swollen lymph nodes. After the patient was admitted to the hospital, the infecting organism was identified as Gram-positive and round in shape. A drug particularly effective against Gram-positive organisms was administered, but no special precautions to prevent spread of the infection were taken. Within six hours, the patient had died. Later investigation revealed that the bacteria in question were Gram-negative, short rods. In fact, the disease in question was identified as plague. More than 30 people were exposed needlessly to possible infection as a result of this laboratory error!

The principle and step-wise procedure of Gram staining are summarized in following Table : B-1.

Table B-1 : A representative standardized Gram-staining procedure

Reagents in their order of application	Reactions Gram- positive	Grain-negative	Appearance G+	G–
Primary stain [crystal violet -CV]	(1) Dye is taken up by cells in two forms, bound and unbound	(1) Same		
	(2) Cells appear violet	(2) Same	Violet	Violet

... contd.

Reagents in their order of application	Reactions Gram- positive	Grain-negative	Appearance G+	G–
A mordant [Gram's Iodine solution – I]	(1) Iodine reacts, i.e. fixes probably both the unbound and bound crystal violet.	(1) same		
	(2) A CV-I precipitate complex is formed.	(2) same		
	(3) Cell remain violet in colour	(3) same	Violet	Violet
Decolourizer [Ethanol (95%) or mixture of acetone-ethanol]	(I) The decolourizer causes the dissociation of CV - I i.e. CV-I = CV + I	(1) same		
	(2) The components of the complex are now soluble	(2) same		
	(3) Dehydration of thick cell wall occurs,	(3) Dehydration of the thin cell envelope occurs.		
	(4)Diffusion of dye proceeds slowly, cells remain violet	(4) Diffusion of dye proceeds faster. cells becomes colourless	Violet	Colourless
Counter stain [Safranin or a dilute solution of carbolfuchsin]	(1) some displacement of CV may occur, but in general cells are not affected.	(1) displacement of any CV left occurs.		
	(2) cells appear purple.	(2) cells take up counterstain.	Purple or	pink or
		(3) Cells appear pink	violet	red

(2) ACID-FAST STAINING : (ZIEHL - NEELSEN TECHNIQUE)

(A) Introduction

Acid-fast staining is an another widely used differential staining procedure for *Mycobacteria*. This technique distinguishes acid-fast bacteria (*Mycobacterium* spp) from non

-acid fast bacteria. Acid-fast means a property of certain bacteria to retain the initial stain (carbol fuchsin) and difficult to decolourize with acid-alcohol.

(B) Theory of staining

Acid - fastness depends on the integrity of the structure of waxy envelope. Acid - fastness property is attributed to the presence of high lipid content in the cell envelope which binds the fuchsin dye so that it is not destained with acid – alcohol. In certain acid - fast bacteria, lipid content is as high as 60 % (w/w) . The major lipids are mycolic acids, glycolipids and mycosides.

The dye basic fuchsin is more soluble in phenol than in water or acid alcohol. Phenol, in turn, is more soluble in lipids that are present in envelope of acid fast bacteria. During staining, basic fuchsin enters the cell. Phenol and heat act as intensifiers. Basic fuchsin is retained in the cell as it is more soluble there than in acid - alcohol, while non-acid fast bacteria are destained and are finally counterstained with the dye of different colour like methylene blue or malachite green.

(C) Examples of Acid - fast Bacteria

Mycolic acids having about 90 (ninety) carbon atoms occur in cell wall of mycobacteria - *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the causative agents of tuberculosis. *Mycobacterium leprae* is the causative agent of leprosy.

Mycobacterium phlei and *Mycobacterium smegmatis* are harmless saprophytes. The genus *Nocardia* contains acid-fast species. Mycolic acids called nocardomycolic acids are present in cell wall of *Nocardia.*

(D) Ziehl-Neelsen staining technique

(1)   Prepare the smear on a clean galss slide from sputum or culture sample. Fix it with heat.

(2)   Cover the smear with a small rectangle filter paper of 2 cm. × 3cm. in size.

(3)   Apply 5 to 7 drops of Z.N.C.F stain solution to saturate the filter paper.

(4)   Place the slide on wire gauze and heat the stain-covered slide till steam rises for about 5 minutes. Add more Z.N.C.F but do not allow slide to dry.

(5)   Cool the slide, remove the filter paper with forcep. Rinse with water to wash off stain.

(6)   Decolourize with acid alcohol or 20% H_2SO_4 , until no more stain appears in washing.

(7)   Wash off excess acid alcohol or 20% H_2SO_4 with water.

(8)   Counterstain with 0.3% methylene blue for 1-2 minutes or with 1% aqueous solution of malachite green for 1 minute.

(9)   Wash it with tap water, and air dry.

(10) Examine under oil-immersion objective lens.

Result—Acid-fast bacteria are stained red, while non-acid fast bacteria are stained blue or green depending upon the counterstain employed.

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Appendix C
ENZYMES USED IN DAIRY INDUSTRY

(1) LACTASE

Milk and whey contain about 4.7% (w/v) lactose. Adults having their origins in Northern Europe and Indian subcontinent are tolerant to lactose. But a great majority of the adults of the remaining regions of the world are intolerant to lactose, e.g. 97%, 90% and 70% of Thai, Chinese and black American populations, respectively. In addition, some rare individuals are intolerant to lactose due to lactase deficiency or some other metabolic defects. Lactose intolerant individuals suffer from diarrhoea, abdominal cramps, flatulence and, in severe cases, dehydration and even death, when they consume normal milk. Clearly such persons need low-lactose milk/milk preparations.

In addition, lactose is poorly soluble in water (crystals form above 11% of w/v at 4°C). This hampers the use of concentrated whey syrups in many food processes, makes the foods prone to microbial attack, and the disposal of such whey is rather expensive (due to the presence of lactose). These problems are readily overcome by hydrolysis of lactose using the enzyme lactase, which produces the monosaccharides D-glucose an β-D-galactose, as shown in following equation:

$$\text{Lactose} + H_2O \xrightarrow{\text{Lactase}} \text{D-glucose} + \beta\text{-D-galactose}$$
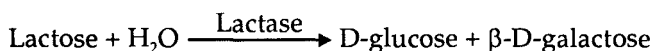
The lactase enzyme is obtained from following sources:

(i) *Kluyveromyces fragilis* (pH optimum of 6.5-7.0 which is suitable for treatment of milk).

(ii) *Aspergillus niger* or *Aspergillus oryzae* (pH optima of 3.0-4.0 and 4.5-6.0 respectively, and more suited for treatment of whey).

Specific uses of Lactases

(1) Lactases are added to milk or whey at about 2,000 U/kg and incubated for 1 day at 5°C; about 50% of the lactose becomes hydrolysed making the milk or whey more sweet. In addition, when the milk/whey is condensed or frozen, lactose does not from crystals.

(2)   Lactase is used in production of ice creams, and sweetened, flavoured and condensed milks.

(3)   Lactase-treated whey powder is used either in part or full, in place of the skimmed milk powder in ice cream recipes; this also improves the creaminess of the ice creams.

(4)   Small amount of lactase (20 U/kg) may be added to ultra-high temperature sterilized milk; storage for one month at 20°C produces low lactose milk.

(2) BETA-GALACTOSIDASE

Beta-galactosidase (E.C. 3.2.1.33) is produced by variety of microorganisms. Commercially available enzymes are derived from *Kluyveromyces lactis* or *Aspergillus niger*. Yeast enzymes function in pH range 6-7 while filamentous fungal enzymes function at pH 4-5. Therefore, yeast enzymes are used for hydrolysis of lactose in milk while fungal enzymes are used for hydrolysis of lactose in whey.

The major applications of beta-galactosidase are found in—

(i)   liquid milk and milk powder, to improve the product for lactose-intolerant individuals, or to increase the sweetness of milk-based drinks;

(ii)   concentrated milk products, to prevent crystalisation of sugars;

(iii)   fermented milk products, to increase fermentation rate;

(iv)   whey as animal feed additive, to increase feed rate intake;

(v)   whey as food ingredient, to increase sweetness and prevent crystalisation (applied in ice-cream, confectionary and bakery products).

Optimal temperature for beta-galactosidase is 35°C and in batch process, hydrolysis of milk proceeds in 4 hours. Alternately hydrolysis at 6°C for 20 hours also will avoid contamination.

Acid hydrolysis of lactose produces unwanted by-products, so enzymatic hydrolysis is preferred.

(3) PROTEASES

Traditionally rennet (a mixture of chymosine and pepsin) is used for curdling of milk. Rennet is obtained by salt extraction of the stomaches of suckling calves. Only chymosine is capable of specific hydrolysis of k-casein which results in curdling of milk. The ratio of pepsin to chymosine increases during ageing of calves. Pepsin can have negative effects on flavour development of the cheese. Rennet containing >15% of pepsin is considered unsuitable for typical Dutch cheese manufacturing.

Proteases from different microorganisms are used for development of flavour in cheese. As there is shortage of calf rennets, alternate proteolytic enzymes are being investigated. Proteases from *Mucor miehei* and *Mucor pusillus* show nearly the same specificity as chymosine. Minor differences in flavour development is observed in long maturation requiring cheeses in rennet and microbial enzymes. Thermostability of microbial enzymes is problem in application. Now by mild oxidation thermostability of microbial enzymes is reduced without affecting original activity and the problem of residual active enzyme and its interference in formulation of dairy products and baby foods from whey is solved.

Recombinant DNA technology was used to transfer calf rennet gene to yeast. This approach is very effective and reduces the need of suckling calves.

(4) LIPASES

Lipases are used along with proteases for preparation of enzyme-modified cheese (EMC). Lipases are also used in certain Italian cheeses to give them their characteristic flavour. In this case enzymes are used to create natural flavour and not for development of flavour concentrate. The enzyme is added to the milk before the addition of rennet and is active under normal ripening conditions.

An example is the use of *Picantase*, a lipase produced by the fungus *Mucor miehei* for the production of *Remano* and *Provolone*.

Brittle fat can also be modified to give a unique flavour. The modified fats are used in confectionary as coffee whitner and in bakery products.

(5) ENZYME-MODIFIED CHEESE (EMC)

Proteases and lipases from different microorganisms are used for the production of cheese flavours. The technology consists of the incubation of the natural cheese slurry with specific proteases and lipases, usually at 37°C for period of 2 days to 2 weeks, followed by inactivation of the enzyme and formulation of the end product into paste.

EMC is available in variety of types (e.g., Cheddar, Parmesan, Edam) and with different strength with regard to the flavour concentrate (e.g., 5 to 20 times the intensity of normal cheese). EMCs are used as cheese substitutes in number of products such as snacks, pizzas and cheese dips.

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Appendix D
EXPLANATION TO IMPORTANT TERMS

1.	Activity Test	:	The test done to check metabolic activity of starter cultures under standard set of conditions, e.g. Rate of acid production.
2.	Antibodies	:	The type of specific immunogenic substances (proteins) produced by the body in presence of a specific antigen.
3.	Bactofugation	:	A process of separation of microbes from milk by centrifugal action.
4.	Bittiness	:	A type of defect produced in cream by phospholipase produced by *B. cereus*, which is characterised by presence of flakes or flecks which float on the top, when cream is added to hot fluids.
5.	Casein	:	A typical major milk protein.
6.	Cheese spread	:	A product made after heat processing of natural cheeses with emulsifying agents which can be used for spreading on bread.
7.	Clean milk	:	The milk drawn from the udders of healthy animal, which is collected in clean, dry utensils, which is free from extraneous matters, which has normal composition and possess natural milk flavour, which has low bacterial count and which is completely safe for human consumption.
8.	Colostrum	:	The milk secreted from the udders immediately after parturition. It differs considerably in composition from the latter secretion.
9.	Early blowing	:	The gas formation in cheese due to coliforms or yeasts in the first few days of ripening.
10.	Eye formation	:	Formation of uniform gas pockets/holes in Swiss cheese blocks due to the activity of Propionibacteria.

11.	Fermented milk	:	Coagulated, set or stirred milk, fermented by means of specific organisms.
12.	Fore milk	:	First few strippings of milk taken from teats, which has relatively high bacterial load.
13.	Intermediate Moisture Food	:	Foods with moisture content between dry foods which can be stored at room temperature and moist foods which need to be frozen, refrigerated, canned or preserved by some other mechanisms. These foods generally have moisture content between 10 to 40% or water activity between 0.65 to 0.90.
14.	Late blowing	:	The gas formation in cheese due to Clostridia after few weeks of ripening.
15.	Lipolysis	:	Break down of fat into glycerides or fatty acids due to action of lipases or esterases.
16.	Mammary gland	:	The gland organ in females secreting milk.
17.	Micro-environment	:	The conditions (nutrients, toxic substances, pH, Eh, moisture, etc.) existing in a very small section immediately adjacent to microorganisms, that decides the fate of those microbes.
18.	Milk	:	The liquid food secreted from the mammary gland of mammalians for the nourishment of their new borns.
19.	Mycotoxin	:	Toxic substances produced by molds.
20.	Phage (Bacteriophage)	:	It is a virus infecting bacteria.
21.	Phagocytosis	:	A process of engulfment and ingestion of particles or parasites by the cell.
22.	Plasmid	:	Extrachromosomal DNA segments present in the cells which are capable of autonomous replication and transfer.
23.	Probiotics	:	The substances or living materials helping the growth and performance of the host.
24.	Processed cheese	:	Cheese obtained by heat processing of natural cheeses in presence of stabilizers, emulsifier, flavouring agents, fat, etc.
25.	Propagation	:	A method of cultivation of microbial cultures for maintenance or increasing the cell numbers and scale up.
26.	Proteolysis	:	A break down of proteins to peptides, amino acids and other compounds due to the action of proteases and peptidases.
27.	Psychrotrophs	:	The organisms capable of growing at 7°C or less, irrespective of their optimum temperature.
28.	Rennet	:	A milk clotting enzyme obtained from abomasum of young calves, used in the manufacture of cheese.

29.	Rennet substitutes	:	Rennet like milk clotting enzymes obtained from plant or microbial sources, used in the manufacture of cheese.
30.	Rind rot	:	Softening, off flavours and discoloration caused on the surface of cheese due to unwanted surface growth of microorganisms.
31.	Ripening	:	Process of aging cheese by storing at controlled temperature and humidity to bring about desirable changes in cheese.
32.	Shelf-life	:	The duration or period for which the product remains stable or can be offered for sale, without deterioration.
33.	Soil	:	Materials like milk residues, water droplets, dust, sediments, detergent or sanitizer residues, etc. adhering to equipment surfaces.
34.	Starter cultures	:	Selected groups of pure and actively growing microorganisms, which are used singly or in combination as inoculum to bring about desirable changes in the medium (milk).
35.	Starter distillate	:	Preparation obtained by distillation of volatile flavour compounds from the liquid starter cultures.
36.	Symbiosis	:	Association of two or more organisms in which the relationship is mutually beneficial.
37.	Thermal Death Time (TDT)	:	It is the time necessary to kill all the organisms under specified conditions at a specified temperature.
38.	Thermodurics	:	A group of microorganisms resisting pasteurization time-temperature combination of milk.
39.	Water activity (a_w)	:	Ratio of equilibrium vapour pressure of the sample to that of pure water at the same temperature. It decides the degree of free water available for the microbial growth in foods.
40.	Whey	:	It is the fluid obtained during manufacture of cheese, paneer or casein, after removal of casein curd, which is rich in water soluble components of milk.
41.	Working	:	A process of kneading butter that helps in making compact fat mass and uniform distribution of moisture.

□□□

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