Unit – I Dairy Microbiology

- **Definition of and composition of milk**

Milk is a whitish liquid containing proteins, fats, lactose, and various vitamins and minerals that is produced by the mammary glands of all mature female mammals after they have given birth and serves as nourishment for their young.
Milk is a white liquid produced by the mammary glands of mammals. It is the primary source of nutrition for young mammals before they are able to digest other types of food. Early-lactation milk contains colostrum, which carries the mother's antibodies to the baby and can reduce the risk of many diseases in the baby.

Milk (cow or buffalo or goat milk) may be defined as the whole, fresh, clean, lacteal secretion obtained by complete milking of one or more healthy animals, practically colostrum free and containing the minimum prescribed percentages of milk fat and milk solids not fat (SNF).

Colostrum (**first milk**) is a form of milk produced by the mammary glands of mammals just prior to giving birth. Colostrum contains antibodies to protect the newborn against disease, as well as being lower in fat and higher in protein than ordinary milk. Colostrum is very rich in proteins, vitamin A, and sodium chloride, but contains lower amounts of carbohydrates, lipids, and potassium than normal milk. The most pertinent bioactive components in colostrum are growth factors and antimicrobial factors. The antibodies in colostrum provide passive immunity, while growth factors stimulate the development of the gut. They are passed to the neonate and provide the first protection against pathogens.

The milk of cows, goats, buffalos or other animals is used as food by humans.

Composition of different species of milk

Species	Percentage of Composition				
	Water	Fat	Protein	Lactose	Ash
Elephant	67.8	19.6	3.1	8.8	0.7
Buffalo	84.2	6.6	3.9	5.2	0.8
Camel	86.5	3.1	4.0	5.6	0.8
Goat	86.5	4.5	3.5	4.7	0.8
Cow	86.6	4.6	3.4	4.9	0.7
Human	87.7	3.6	1.8	6.8	0.1

Milk is a white or yellow-white, opaque liquid. The color is influenced by scattering and absorption of light by milk globules and protein micelles. A yellowish, color is derived from **carotene** present in that fat phase and from **riboflavin** present in the aqueous phase. Milk tastes mildly sweet, while its odor and flavor are normally quite faint. Milk occurs in the form of droplets or globules, surrounded by a membrane and emulsified in milk serum (also called whey).

The fat globules (called cream) separate after prolonged storage or after centrifugation. The fat globules float on the skim milk. **Homogenization** of milk so finely divides and emulsifies the fat globules that **cream separation does not occur** even after prolonged standing. Proteins of various sizes are dispersed in milk serum. They are called micelles and consist mostly of calcium salts of casein molecules. Furthermore, milk contains lipoprotein particles, also called milk microsomes, which consist of the residue of cell membranes, microvilli, etc, as well as somatic cells which are mainly leucocytes.

Chemical composition of Milk

Various carbohydrates, fats, proteins, minerals and other ingredients are solubilized in milk serum.

Carbohydrates

Lactose is the **major carbohydrate** in the milk of most species. Lactose is a **disaccharide** composed of the monosaccharides D-glucose and D-galactose, joined in a β-1,4-glycosidic linkage. Lactose is cleaved to glucose and galactose in the intestine of the neonate by an enzyme activity called **lactase** (or β-galactosidase). The galactose is then converted to glucose by a different enzyme. Lactose is a major, readily digestible source of glucose which

provides energy for the neonate. Lactose intolerance can occur in adult animals or animals that do not have lactase activity in their intestines. Other carbohydrates are found in milk, but at low concentrations. Other carbohydrates found free in milk include glucose, amino sugars, sugar phosphates, neutral and acid oligosaccharides, and nucleotide sugars.

Fats

Milk fat is composed of a complex mixture of lipids. **Triglycerides** are the major type of lipid in milk fat. Triglycerides are composed of three **fatty acids** covalently bound to a **glycerol** molecule by ester bonds. Milk fat is the major source of lipid used by the neonate mammal for accumulating **body adipose** in the initial days after birth.

Proteins

The total protein component of milk is composed of numerous specific proteins. The primary groups of milk proteins are the **caseins**. There are 3 or 4 caseins in the milk of most species; the different caseins are distinct molecules but are similar in structure. All other proteins found in milk are grouped together under the name of **whey proteins**.

The major whey proteins in cow milk are **beta-lactoglobulin** and **alpha-lactalbumin** are synthesized in the mammary epithelial cells and are only produced by the mammary gland. The immunoglobulin and serum albumin in milk are not synthesized by the epithelial cells. Instead, they are absorbed from the blood.

Caseins are composed of several similar proteins which form a multi-molecular, granular structure called a **casein micelle**. In addition to casein molecules, the casein micelle contains water and salts (mainly calcium and phosphorous). Some enzymes are associated with casein micelles, too. In the stomach of the young of many species is an enzyme called **rennin** which specifically hydrolyzes part of the casein micelle resulting in formation of a **curd**. Caseins are highly digestible in the intestine and are a high quality source of amino acids. Most whey proteins are relatively less digestible in the intestine, although all of them are digested to some degree.

Water

Water content of milk is dependent upon the synthesis of lactose. Without some water in the milk, milk would be a viscous secretion composed mostly of lipid and protein and would be extremely difficult to remove from the gland.

Minerals

Calcium and **phosphorous** are the major minerals found in milk. These minerals are required in large quantities by the rapidly growing neonate for bone growth and development of soft tissues. Calcium and phosphorous mostly are associated with the casein micelle structure. Milk also contains most other minerals found in the body.

Some minerals, such as Zn, Mg, Fe, Cu, Mn, and Mo, are required by enzymes as cofactors. Minerals contribute to the buffering capacity of milk, the maintenance of milk pH, the ionic strength of milk, and milk's osmotic pressure.

Trace Elements

The milk concentration of some elements can be increased by increasing the amount in the diet. These particularly include I, B, Br, Co, Mn, Mo, Se, and Zn.

Vitamins

Milk contains all the **vitamins** except Vit C required by mammals. The **fat soluble vitamins**, A, D, E, and K, are found primarily in the milk fat; milk has limited amounts of vitamin K. The **B vitamins** are found in the aqueous phase of milk.

Vitamins are essential organic compounds required in the diet. The fat soluble vitamins (A, D, E, K) are associated with the milk fat globule.

Somatic cells

Milk also contains a range of other components. Milk contains leukocyte cells, also known as **somatic cells** in cow milk

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- **<u>Sources of Microorganisms in Milk –</u>**

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.

1. **Producing Animal**
2. **Milking Area**
3. **Utensils and Equipments**
4. **Personnel (Milking men)**
5. **Water**

1. **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 a 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 diseases 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. 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 harbors organisms. The hair, dirt and dust often fall from the body into the milking pots or the teat cups of milking machines. The modern practice is to keep the flanks clipped to minimize contamination.

2. **Milking Area** –

The microbial content of air is greatly affected by many conditions and practices. Dried dirt and waste is picked up by all 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.

3. **Utensils and Equipments-**

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 milliliter. Pails (containers, buckets), strainers (sieve), milking machines, cans, pipes, bottles, and other equipments used for the handling of milk are sometimes not properly washed and sanitized.

Organisms survive in the cracks, corners, crevices (gaps), dents (cavity), 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.

4. **Personnel (Milking men)**

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.

5. **Water**

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.

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- **<u>Desirable changes carried out by microorganism in milk</u>**

Fermented Dairy Products - Many products are made through microbial fermentation of milk, including buttermilk, yogurt and many cheeses. Fermentation is primarily carried out by lactic acid bacteria.

1. **Buttermilk, Curd, Sour cream, Kefir and Kumis**.

   These are fermented milk products produced by *lactobacilli* and *leuconostoc species.* Sour cream uses *Streptococcus cremoris* or *S. lactis* for producing lactic acid and *Leuconostoc cremoris* for characteristic

flavour. Cream is starting substrate. Butter is normally made by churning cream that has been soured by lactic acid bacteria.

2. **Yogurt.**

   It is made by fermenting milk with a mixture of *Lactobacillus bulgaricus and Streptococcus thermophilus* at 40°C. Flavour is due to accumulation of lactic acid and acetaldehyde.
3. **Cheese.**

   Cheese consists of milk curds that have been separated from the liquid portion of the milk (whey). The curdling of milk is done by enzyme rennin (casein coagulase or chymosin) and lactic acid bacterial starter cultures. Cheeses are classified as soft (high, 50-80% water content), semi hard (about 45% water) and hard (a low water content, less than 40%).

- **<u>Undesirable changes carried out by microorganism in milk</u>**

Spoilage occurs when microorganisms degrade the carbohydrates, proteins, fats of milk and produce noxious, end products. Milk products as follows;

1. **Gas Production**
2. **Ropiness / sliminess**
3. **Proteolysis**
4. **Lipolysis**
5. **Sweet curdling**
6. **Bitty Cream (or) Broken cream**
7. **Development of abnormal Flavours**
8. **Development of abnormal colour fermentations**

1. Gas Production:

The production of gas mainly CO_2 by certain organisms in dairy products is responsible for a defect called **"gassiness".** In some cases it is associated with acid production. In high fat milk there is foaming and the gas escapes the partially coagulated mass and the defect is called **"Frothiness".** This is due to associative action of acid producing bacteria with yeasts. The production of gas

in canned dairy products causes bulging of cans and the defect is called **'blowing**' of cans.

Causative organisms:

i. **Coliforms:** *E. coli, Enterobacter aerogenes* ferment lactose of milk or cream into gas and acid. These are called 'early gas producer' and produce early blowing condition.

ii. **Anaerobic spore forming bacteria:** ex. *Cl. butyricum, Cl. sporogenes*— produce gas only in anaerobic conditions, mostly in canned dairy products like processed cheese, concentrated milk. They are called "Late gas producers' and produce late blowing condition.

iii. **Lactose fermenting yeasts:** ex. *Candida psedutropicalis*, yeasts produce CO_2 and small amounts of ethyl alcohol in milk and cream, whey at or below 37°C.

2. Ropiness / sliminess:

Ropy fermentation is brought about by the growth of bacteria leading to change in consistency of the produce that forms threads of viscous masses when poured. Ropiness develops only on storage and milk is drawn out as fine threads and may appear gel like consistency. Sometimes the change is so much pronounced that the milk can be drawn into long thread.

Causative organisms:

i. **Gram –ve rods**: *Alcaligenes viscosus*
ii. **Coli-aerogenes group**: This group consists of ropy strains belonging to *enterobacter, citrobacter*, *Serratia marcescens* and related genera,
iii. **Aerobic spore formers**: *B. cereus, B. subtilis, B. circulans*
iv. *S. lactics var hollandicus, L. casei, Lactobacillus delbruckeii ssp bulgaricus* show ropiness before detectable acid development. Ropiness decreases as acidity increases.

Mechanism of ropiness:

True gums or gum like substances which are polysaccharides. Gums are galactans produced by fermentation of lactose. **Mucins** – Nitrogenous mucous like substance. Peptonizing bacteria produce mucins which are combination of proteins with carbohydrate radical. **Exopolysaccharides** produced as capsules associated with cell (or) as slime unattached to the cell. *Al. viscosus* produces capsular materials.

3. **Proteolysis**:

It is the process by which casein or some insoluble casein derivatives are broken down to water soluble compounds through the action of organisms or their enzymes. It is of significance because of loss of quality of products due to proteolytic spoilage.

Causative organisms:

Psychotrophs are the actively proteolytic organisms and grow at 7°C or less especially *Pseudomonas fluorescens, Ps. fragi, Alteromonas putrefaciens*. Thermoduric bacteria especially *Micrococcus caseolyticus, B. stearothermophilus B. cereus, B. subtilis.*

Proteins
↓ Proteinases
Proteoses
↓ Proteases
Peptones
↓ Proteases
Polypeptides
↓ polypeptidases
Peptides
↓Dipeptidases
Amino acids

4. **Lipolysis**:

Lipolysis is the hydrolysis of milk fat by lipases resulting into the accumulation of free fatty acids. The lower chain FFA particularly butyric and

caproic are responsible for the lipolytic off flavours, also referred to as rancidity (Hydrolytic rancidity).

Lipolytic microbes or enzymes:

a) Psychrotrophes: Pseudomonas sp. mainly *Ps. fragi, Ps. fluoresens, Achromobacter lipolyticum*

b) Other types

Micrococcus frendenreichii, Bacillus cereus, B. subtilis, B.coagulans

c) Yeasts & molds

C. lipolytica, Geotricum candidum, Penicillium spp. and Aspergillus spp.

5. Sweet curdling

Curdling without pronounced acid production is called the sweet curdling. The defect is due to the production of an extracellular enzyme similar to rennin by bacteria which causes casein to precipitate in the term of small specks of curd before the development of sufficient acidity i.e between 6.2 and 6.6 pH.

Causative organisms:

i. Cocci: *S. liquifaciens*

ii. Aerobic spore formers – *B. cereus, B. subtilis*

iii. Psychrophilic spore forms- *B. cereus, B. licheniformis, and certain Microbacterium spp.*

iv. Non spore forming rods: *Proteus and Escherichia*

6. Bitty Cream (or) Broken cream:

It is characterized by the appearance of flakes in the cream which do not mix again when milk is shaken. If such milk is used in tea, the flakes float on the surface making it unaccepted to mainly people. Flaking normally occurs before the changes in flavour or heat stability.

Bacteria origin – Produced partly by the lecithinase enzyme of *B. cereus.*

Bitty cream is the chief spoilage problem of pasteurized milk. Failure of refrigeration, Seasonal variation prolonged storage etc., are the main reasons.

7. Development of abnormal Flavours

a. **Fruity Flavours**: These are due to ethyl ester formation usually catalyzed by esterases from psychrotroph or lactic acid bacteria. Ester formation by *Ps. fragi* involves liberation of butyric & caproic acids from one and three positions of milk triglycerides and are esterified with ethanol. Predominate esters are Ethyl butyrate, Ethyl hexanoate.

b. **Malty flavour**: Caused by Malty strains *Lactococcus lactis* sp

c. **Bitty flavour:** Caused by proteolytic organisms especially *Bacillus sp.*, and *Pseudomonas sp.*

d. **Fishy flavour:** Caused by *Ps. icthyosmius*

e. **Potato flavor:** Caused by *Ps. mucidolens* and *Ps. graveolens*

f. **Phenolic flavour:** Caused by *Bacillus circulans*

g. **Soapy flavour:** Caused by Ps. sapoticum

h. **Bitty/Musty flavour:** Caused by *Actinomyces and certain yeast*

i. **Burnt of caramel flavour:** Caused by Malty strains of *Lactococcus lactis*

j. **Barny flavour:** Caused by *Aerobacter oxytocum*

8. Development of abnormal colour fermentations:

a. **Yellow coloration**: *Pseudomonas synxantha*

b. **Blue coloration:** *Pseudomonas cyanogens*

c. **Green coloration:** *Penicillium roqueforte*

d. **Black coloration:** *Pseudomonas nigrifaciens*

e. **Red coloration:** *Serratia marcescens/Micrococcus resen*

f. **Brown coloration:** *Pseudomonas fluorescens*

g. **Greenish coloration:** *Pseudomonas fluorescens*

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- **Types of microorganisms – Biochemical types, temperature characteristic & pathogens**

Types of Microorganisms in Milk - The types of micro organisms found in milk vary considerably, and are dependent upon the specific conditions associated with a batch of milk.

Bacteria, yeasts, molds, and bacteriophages are commonly encountered. Other viruses and Protozoa are seldom observed in milk products, except as occasional contaminants.

Bacteria

Bacteria are the most common, and probably the most numerous of microorganisms with which the dairy processing industry is concerned. They belong to four main groups: (1) cocci, usually gram positive, (2) gram positive non-sporeforming rods, (3) gram positive sporeforming rods, and (4) gram negative non-spore forming rods.

Yeasts

The yeasts most frequently encountered in milk and milk products act upon the lactose to produce acid and carbon dioxide. Yeasts are more commonly found in raw, cream during hot weather, but are potential contaminants throughout the year.

Molds

Molds often grow in large concentrations and are visible as a fuzzy or fluffy growth. They are sometimes observed on the surface, of butter, old cream, khoa, or cheese. They are black, grey, green, blue or white. They discolour milk products and often produce undesirable, at times repulsive, odours and flavours. Moulds are essential in production of the certain kinds of cheese.

Bacteriophages

Bacteriophages are particularly obnoxious in starter cultures used for making cultured milk, butter and cheese. Phages will, kill the bacterial culture and the whole process of fermentation will be lost.

Microorganisms found in milk can also be described on the basis of the following characteristics.

1. Biochemical activities.

2. Temperature response.

3. Ability to cause infection and disease (bovine and human origin)

1. Biochemical activities

If allowed to stand under condition that permits bacterial growth, raw milk of a good sanitary quality will rapidly undergo a series of chemical changes. The principal change is lactose fermentation to lactic acid.

i) **Acid producing: -**This change is brought about by aciduric lactic organisms, especially *Strepotococcus lactis* and certain lactobacilli. These include two distinct biochemical types, homo-and heterofermentative. In homofermentation lactic acid is the major product of lactose fermentation. Heterofermentative organisms, however, produce lactic, acetic, propionic, and some other acids, and some alcohols and gases such as CO_2 and H_2. Organisms continue to form lactic acid until the concentration of acid is itself too great for the organisms to remain live.

Microbacteria, micrococci, coliforms, etc. also ferment lactose to lactic acid and other products. Many Clostridium species and, some yeasts such as *Torula lactic*, and *Torula cremoris* ferment lactose with acid and gas production. As the acidity continues to increase and reaches a pH of 4.7, it eventually causes a precipitation of casein. Organisms capable of metabolizing lactic and other acids develop especially aciduric, yeasts and molds.

The acidity of milk is diminished and the alkaline products of protein decomposition such as amines, ammonia and the like are produced. This is accomplished by many species of the genera Bacillus, Clostridium, Pseudomonas, Proteus and numerous other forms.

ii) Proteolytic:

These microorganisms degrade casein or some insoluble casein derivatives to water soluble compounds through the action of organisms or their enzymes.
E. g. Pseudomonas fluorescens, Ps. fragi, Alteromonas putrefaciens. Thermoduric bacteria especially *Micrococcus caseolyticus, B. stearothermophilus B. cereus, B. subtilis.*

iii) Lipolytic: -The action of microorganisms does not involve fat as readily as it does lactose and protein. Lipolysis results from the action of lipase produced by bacteria such as Pseudomonas, Achromobacter and by some yeasts and molds. Fat is hydrolyzed to glycerol and fatty acids. Some of the fatty acids, for example, butyric and caproic acid give milk products, distinctive and usually rancid, odours and flavours.

iv) Ropiness producing: -Several microorganisms also bring about certain objectionable changes in the milk which may not be deleterious to health. Ropines in milk are sometimes encountered. The milk become ropy or slimy and may be pulled out into long threads. It is produced by several organisms but the most important species is *Alcaligenes viscolactis*. A rapid fermentation of lactose in milk is sometimes observed and is known as stormy fermentation. This is brought about by *Clostridium perfringens*.

v) Gas producing: - The curd becomes torn to shreds by the vigorous fermentation and gas production.

vi) Colour producing: - Several organisms have been isolated from milk which imparts brilliant colours. *Pseudomonas syncyanea* imparts blue colour, *pseudomonas synxantha* yellow colour and *Serratia marcescens* red colour to the milk.

2. Temperature response

Microorganisms found in milk can also be described according their optimum temperature for growth and heat resistance. This is a very practical consideration since milk is preserved by employing low temperatures to prevent changes due to microbial activity and by high temperatures to reduce microbial population and destroy pathogens. All the four types of microorganisms i.e. psychrophilic, mesophilic, thermophilic and thermoduric are found in milk.

i) Psychrophiles grow at temperatures just above freezing and at refrigeration temperatures. They produce a wide variety of spoilage defects. The defects may result in the production of many "off" flavours and odours. The most commonly encountered psychrophilic bacteria are members of the genera pseudomonas, Achromobacter, Vibrio, Flavobacterium and Alcaligenes, They are killed in the pasteurization process, but are sometimes found in pasteurized milk. The contamination takes place after pasteurization from equipment, cans, bottles, and water.

ii) Mesophilic: -The most important mesophilic bacteria are streptococci, lactobacilli and coliforms, which produce acid and gas and off flavours. They are killed in the pasteurization process.

iii) Thermophilic bacteria grow well at the temperature used in pasteurization, especially when the low temperature holding method is followed. Most thermophilic forms are found in two genera, *Bacillus and Clostridium.*

iv) **Thermoduric** organisms are regarded as those which survive pasteurization but do not grow at pasteurization temperatures. The most common thermoduric bacteria are found in the genera *Microbacterium, Corynebacterium, Micrococcus, Streptococcus and Bacillus.*

3. Ability to cause infection and disease (bovine and human origin)

Pathogenic organisms of both bovine and human origin have been isolated from milk. Milk, therefore, can serve as a carrier of diseases. Many serious epidemics were caused by the consumption of such products before this fact was clearly recognized. However, this became less common as milk sanitation has improved and pasteurization is being more widely practiced.

The disease organisms present in milk may be derived from

(1) Diseased animals or

(2) Persons collecting and handling milk

Thus the danger is due to the inoculums and not to the growth of organisms in the milk. The health of animal is an important factor. Several diseases of cattle including staphylococcal and streptococcal infections, tuberculosis, brucellosis, salmonellosis, Q fever and Foot and mouth disease

may be transmitted to man. The organisms causing these diseases may get into the milk either directly from the udder, or indirectly from infected body discharges, which may drop, splash, or be blown into the milk.

Some of the important diseases of human origin that have been transmitted by milk are

(1) Typhoid fever	*Salmonella typhi*
(2) Diphtheria,	*Corynebacterium diptheriae*
(3) Scarlet fever,	*Streptococcus pyogenes*
(4) Dysentery	*Shigella dysenteriae*
(5) Septic sore throat	Hemolytic streptococci
(6) Poliomyelitis.	PolioVirus
(7) Tuberculosis,	*Mycobacterium bovis*
(8) Brucellosis	Gram-negative rod *Brucella abortus*
(9) Q fever.	*Coxiella burnetii* (Rickettsia)
(10) Pneumonia,	*Diplococcus pneumonia* and Viruses
(11) Toxoplasmosis,	*Toxoplasma gondii* (Protozoan)
(12) Anthrax,	*Bacillus anthracis*
(13) Foot and Mouth disease	F & M virus
(14) Hepatitis	Hepatitis virus

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- **Changes in the flora of raw milk stored at room temp**

Raw milk contains many types of microorganisms coming from different sources. Microbial spoilage of raw milk can potentially occur from the metabolism of lactose, proteinaceous compound, fatty acids (unsaturated), and the hydrolysis of triglycerides.

If the milk is refrigerated immediately following milking and stored for days, the spoilage will be predominantly caused by the Gram-negative psychrotrophic rods, such as *Pseudomonas, Alcaligenes, Flavobacterium spp*., and some coliforms. *Pseudomonas* and related species, being lactose-negative, will metabolize proteinaceous compounds to change the normal flavor of milk to bitter, fruity, or unclean. The growth of lactose-positive coliforms will produce lactic, acetic, and formic acids, $C0_2$, and H_2 leading to curdling and souring of milk.

Some *Alcaligenes spp* and coliforms can also cause ropiness (sliminess) due to production of viscous polysaccharides. However, if the raw milk is not refrigerated soon, growth of mesophiles predominates e.g, *Lactococcus, Lactobacillus, Enterococcus, Bacillus,* and coliforms, along with *Pseudomonas, Proteus*, and others causing changes like souring and curdling of milk. Yeast and mold growth, under normal conditions, is generally not expected.

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- **Microbiological examination of milk**

There are different approaches for checking the bacteriological quality of raw milk and they are broadly classified into

1) **Direct tests**
   A) Direct Microscopic Count (DMC)
   B) Standard Plate Count (SPC) / Pour Plate Technique or Colony Count Test
2) **Indirect tests such as dye reduction tests**
   A) Methylene blue reduction time test (MBRT test)
   B) Resazurin Reduction test (RR test)
3) **Enumeration of Coliforms in Milk**

1. DIRECT METHODS

A. Direct Microscopic Count test (DMC)
B. Standard plate count test (SPC)

A) Direct Microscopic Count test (DMC)

Microbial count of milk can be determined by direct microscopic examination of milk in a stained smear. In order to express the count quantitatively the area of the microscopic field must be known. The diameter of the oil immersion field is 0.16 mm.

Area of oil immersion field = Πr^2

$= 3.14 \text{ X } (0.08)^2$

$= 0.02$ sq. mm

Thus in 1 cm^2 area number of microscopic fields = 100 / 0.02

($1cm^2$ = 10 X 10 mm= 100) = 5000

The 10 microscopic fields are observed for number of microorganisms and average number of microorganisms per field is calculated. This number is multiplied by 5000 which gives number of organisms in 0.01 ml of milk sample.

Newman's stain is used to remove the milk fat, fix the smear and stain the bacteria in a single operation. The tetrachloroethane of the stain helps to dissolve the milk fat globules, ethyl alcohol fixes the smear and methylene blue stains the smear.

OR Xylene is used to remove fat and then methylene blue is used to stain bacteria.

Advantages of DMC: -

1) It is more rapid. More samples can be examined, as less work is required.

2) The equipments necessary are much less.

3) The slide may be preserved as permanent record.

4) Different morphological types can be distinguished. This information is of great value in determining the source and nature of contamination.

Disadvantages of DMC: -

1) This method may be the source of considerable error unless the food contains a high count. This is because a large factor is used for converting the number of organisms per field to the number per ml of food.

2) This method does not differentiate dead from living organisms.

Requirements: -

1) Milk sample
2) Slide
3) Microscope
4) Newman's Stain (Tetrachloroethane, Ehyl alcohol, Methylene Blue) OR
5) Methylene blue
6) Xylene

Procedure: -

1) 1 sq. cm area is marked on a clean grease free slide with glass marking pencil.

2) 0.01 ml milk sample is spread on opposite side of the marked area.

3) Sample is air-dried and heat fixed.

4) The smear is flooded with Newman's stain for 1 minute, gently washed with tap water, air dried.

OR

5) The smear is flooded with xylene for 1 minute to remove fat from the milk sample. Smear is stained with methylene blue solution for one minute and gently washed with tap water, air dried

6) 10 fields are observed under oil immersion lens.

Direct microscopic counts per ml	**Bacteriological quality of milk**
Less than 5, 00,000	Good
5, 00,000 to 40, 00,000	Fair
40, 00,000 to 2, 00, 00,000	Poor
Over 2, 00, 00,000	Very poor / bad

B. Standard plate count test (SPC) / Pour Plate Technique or Colony Count Test:

The standard plate count method is also called as pour plate technique or colony count test. It is useful in the estimation of number of viable microorganisms in the given sample of milk. The test employs the serial dilution technique for easy quantification of the organisms in view of a wide range of bacterial population that may occur in milk. The appropriate dilutions of the milk sample are mixed with a sterile nutrient medium that can support the growth of the organisms when incubated at a suitable temperature. Each bacterial colony that develops on the plate is presumed to have grown from one bacterium in the inoculum. The total number of colonies counted on the plates multiplied by the dilution factor is taken to represent number of viable organisms present in the sample.

Preparation of dilutions of the milk sample:

Transfer 1ml of the milk sample with a sterile pipette to 9ml of sterile water (1st dilution) which will make 1 in 10 dilution of the milk sample. Take 1ml from 1st dilution and transfer to second 9 ml sterile water to get 1 in 100 dilutions. Mix thoroughly and transfer 1ml from second dilution to third 9 ml sterile water to make 1 in 1000 dilution and so on till a series of required dilutions of the sample is ready. Use a fresh sterile pipette for each successive dilution.

Plating the sample and preparation of plates:

Transfer 1ml of each required dilution into sterile petri dish. To each petri dish add 15 to 20 ml of sterilized Nutrient Agar which was previously sterilized, melted and cooled to 45°C. Mix well, allow the agar to cool and set. Invert the plates and incubate at 37^0 C for 24 - 48 hours. After the incubation determine the average of the counts in the two plates and multiply this by the dilution factor.

Raw milk is graded based on the following specifications

SPC/ml	**Grade**
Not exceeding 2,00,000	Very good
2,000,00 to10,00,000	Good
10,00,000 to 50,00,000	Fair
Over 50,00,000	Poor

Pasteurized milk: A standard plate count of lower than 50,000 Colony Forming Units (CFU) per ml of pasteurized milk is indicative satisfactory quality.

2. INDIRECT METHODS FOR GRADING THE RAW MILK:

Dye reduction tests are the indirect methods of assessing the microbiological quality of milk. They are based on the metabolic activity of the microbes and rely on the oxidation reduction potential of milk. In the tests a correlation is made between the time required for the reduction of the dye and probable bacterial population of milk.

The bacteria present in the milk multiply and consume oxygen for their metabolic activity. The rate of depletion of oxygen influences the oxidation-reduction potential and depends on the number and type of bacteria. The bacterial activity is dependent on the dehydrogenase enzymes, which are flavin enzymes, which can transfer hydrogen / electron from the organic substrate to biological acceptors or in their absence to the reduction dyes. With the depletion of oxygen the redox potential of milk gets reduced to such an extent where it causes the reduction of the dye changing its colour.

C) **Methylene Blue Reduction Time Test (MBRT test)**
D) **Resazurin Reduction test (RR test)**

A) Methylene Blue Reduction Time Test (MBRT test)

The test is useful in assessing the bacteriological quality of milk by determination of the time taken for the reduction of methylene blue in milk indicated by its colour change. Milk in udder has a very low oxidation-reduction potential which rises to + 0.3 volts during the process of milking, storing and cooling due to the incorporation of oxygen. At this potential, the methylene blue will be in oxidized form and have a blue color. The dye gets reduced when the Oxidation-Reduction potential is decreased to = 0.06 to - 0.01 volts due to the depletion of oxygen from milk as a result of metabolic activity of the microrganisms.

The greater is the number of microorganisms in milk, the greater is the metabolic activity and the faster is the reduction of methylene blue and vice-versa.

Requirements: -

2. Milk sample
3. 3 sterile test tubes
4. Methylene blue solution (1 : 2,50,000)
5. Incubator / Water bath adjusted at 37^0 C

Procedure: -

1. Three sterile test tubes are taken.
2. In the first test tube 1 ml methylene blue solution and 10 ml milk sample is added. This test tube is labeled as 'Test'.
3. In a second test tube 10 ml milk sample is taken and it is boiled to kill microorganisms, cooled and 1 ml methylene blue is added to it. This test tube is labeled as 'Negative Control'.
4. In the third test tube 10 ml milk sample is taken and 1ml distilled water is added. This test tube is labeled as 'Positive Control'.
5. All 3 test tubes are shaken well and kept in incubator or in water bath adjusted at 37^0 C.
6. The observations are done after every 15 minutes up to complete disappearance of blue colour or up to 8 hours from the tube labeled as 'Test'. For this observation positive and negative controls are compared.

The quality of raw milk is judged by using the following specifications

Sr. No.	Time required for decolorization of methylene blue	Quality of milk
1.	6 to 8 hours	Excellent / Very good
2.	4 to 6 hours	Good
3.	2 to 4 hours	Fair
4.	Less than 2 hours	Bad / Poor

B. Resazurin reduction test

The principle of this test is same as that of methylene blue reduction test. However, unlike methylene blue the resazurin dye undergoes reduction through

a series of colour shades viz., blue, purple, lavender and pink before completely getting reduced to colourless.

The reszurin dye which is blue in colour at the oxidation-reduction potential of + 0.3 volts changes into pink colour compound (resorufin) as the redox potential reduces to + 0.2 volts. This reaction is irreversible. However the colour of dye changes to colourless (dihydro resorufin) when the redox potential is reduced to +0.1 or less.

Usually the degree of reduction of the dye is measured after a fixed time of incubation of the milk sample in the presence of dye. The reduction of the dye to a particular shade of colour is dependent upon the extent of depletion of oxygen by the metabolic activity of microorganisms. The colour change is measured with the help of a standard colour disc.

Requirements

i) **Standard solution of Resazurin**: Dissolve 0.05 g of resazurin powder in 100 ml of distilled water and boil the contents for 1/2 hour. This will make a standard solution of 0.05%, which should be always kept, in a cool and dark place stored in an amber colored bottle. The bench solution (0.005%) for regular use should be prepared freshly by diluting the standard solution with distilled water i.e. 1 ml of standard solution with 10 ml of distilled water.

ii) Milk Sample

Procedure

i) 1 ml of working solution of resazurin solution and 10 ml of milk is added into a test tube. This test tube is shaken well to mix the milk and Resazurin solution.
ii) This test tube is kept in a thermostatically maintained water bath or in incubator at 37^0 C and the time of incubation is noted down. At the end of one hour of incubation the colour of the milk is compared with one of the colour standards of resazurin disc.

Colour of milk in 1 hour	**Quality of milk**
Blue	Excellent / Very Good
Purple	Good
Lavender	Fair
Pink	Poor
Colourless	Bad / Very Poor

3. Enumeration of Coliforms in Milk:

Coliforms are Gram's negative, oxidase negative, non-spore forming rods which can grow aerobically or facultatively in presence of bile salts or surface active agents with similar growth inhibitory properties and are able to ferment lactose with the production of acid and gas within 48 hours at 37°C.

Their presence in milk or milk products is indicative of possible faecal contamination and is found especially when they are handled under unsanitary conditions. The presence of these organisms is considered undesirable because they produce acid, gas and objectionable taints in the milk products. Generally coliform organisms are destroyed during pasteurization. Their presence in pasteurized milk indicates post pasteurization contamination or improper pasteurization

The test is chiefly based on the principle that the members of this group are capable of producing acid and gas from lactose in the presence of bile salts. Presence of typical coliform colonies in MacConkey,s Agar plates is taken as evidence of coliform contamination. However, sometimes false positives may arise due to the growth of other types of microorganisms such as Clostridium, Bacillus and certain yeasts. In case of doubtful cases, the completed test is commonly employed to confirm the presence of coliforms by using Endo Agar, EMB Agar or BGLBB tubes.

Interpretation:

Absence of coliforms in 1: 100 dilution (less than 100 per ml) in raw milk and in 1:10 dilution (less than 10 per ml) of pasteurized milk is accepted as criterion of satisfactory quality.

MPN Method:

To obtain the number of organisms present in milk a technique called Most Probable Number method. The tubes of lactose broth or MacConkey's broth inoculated with samples of milk are being tested and a count of the number of tubes, showing acid and gas production, is made and the figure is compared to MPN (statistical) table.

- **Pasteurization of milk**

Introduction

The process of pasteurization was named after **Louis Pasteur** who discovered that spoilage organisms could be inactivated in wine by applying heat at temperatures below its boiling point. The process was later applied to milk and remains the most important operation in the processing of milk.

Definition:

The heating of every particle of milk or milk product to a specific temperature below the boiling point for a specified period of time to kill microorganisms that could cause disease, spoilage, or undesired fermentation and to destroy some undesirable enzymes without allowing recontamination of that milk or milk product during and after the heat treatment process.

- It is important to note that **pasteurization is not sterilization**.

Purpose: There are two distinct purposes for the process of milk pasteurization:

1. **Public Health Aspect** - to make milk and milk products safe for human consumption by destroying all bacteria that may be harmful to health (pathogens).
2. **Keeping Quality Aspect** - to improve the keeping quality of milk and milk products. Pasteurization can destroy some undesirable enzymes and many spoilage bacteria. Shelf life can be 7, 10, 14 or up to 16 days.

Time temperature determination: -

Pasteurization conditions were adjusted to 143°F (61.7°C) for 30 minutes or 160°F (71.1°C) for 15 seconds to inactivate *Mycobacterium bovis*, the organism responsible for tuberculosis. However, in 1957 these conditions were shown to be inadequate for the inactivation of *Coxiella burnetii* which causes Q fever in humans. New pasteurization conditions of 145°F (62.8°C) for 30 minutes for a batch process (LTH), or 161°F (71.7°C) for 15 sec for a continuous process (HTST), were adopted in order to inactivate *Coxiella burnetii*, and these conditions are still in use today.

- **Methods for pasteurization**

There are three methods for pasteurizing milk.

1. Low-temperature holding (LTH) method or Batch method.
2. High-temperature short-time (HTST) or Continuous Method or flash pasteurization.
3. Ultrapasteurization (UP).

1. Low-temperature holding (LTH) method or Batch method

In the first method, milk is heated to 145°F (62.8°C) for thirty minutes.

The batch method uses a vat pasteurizer which consists of a jacketed vat surrounded by either circulating water, steam or heating coils of water or steam. In the vat the milk is heated to 62.8^0 C and held for 30 minutes followed by rapid cooling. It is held throughout the holding period while being agitated. The milk may be cooled in the vat or removed hot after the holding time is completed for every particle. As a modification, the milk may be partially heated in tubular or plate heater before entering the vat.

2. High-Temperature Short-Time (HTST) or Continuous Method or Flash pasteurization

In this method, milk is exposed to a temperature of 161°F (71.7°C) for fifteen seconds. This technique is also known as flash pasteurization.

Continuous process method has several advantages over the vat method, the most important being time and energy saving. For most continuous processing, a high temperature short time (HTST) pasteurizer is used. The milk is heated to a temperature of 71.7^0 C and held for 15 seconds and then cooled rapidly. This method is faster and more energy efficient than batch pasteurization.

Here are the basics of HTST:

i. Cold raw milk (39.2^0 F and 4^0 C) is fed into the pasteurization plant.
ii. The milk passes into the regenerative heating section of the plate heat exchanger. The plate heat exchanger is basically a series of stainless steel plates stacked together with some space in between, forming chambers to hold the milk as it passes through. Let's call the odd-numbered chambers "A" chambers, and the even-numbered chambers, "B" chambers. In the

regenerating section, cold milk is pumped through the A chambers, while milk that has already been heated and pasteurized is pumped through the B chambers. The heat from the hot milk passes to the cold milk through the steel plates. This warms the milk to 134.6 to 154.4^0 F (57 to 68^0 C).

iii. Next, the milk passes into the heating section of the plate heat exchanger. Here, hot water in the B chambers heats the milk to at least 161.6^0 F (72^0 C). This is the goal temperature for HTST pasteurization.

iv. The hot milk is then passed through a holding tube. It takes the milk about 15 seconds to pass through the tube, fulfilling the time requirement for this method of pasteurization. The milk has been officially pasteurized once it passes through the holding tube.

v. Now the pasteurized milk is sent back through the re-generative section, where it warms the incoming cold milk. This cools the pasteurized milk to about 89.6^0 F (32^0 C).

vi. In the last part of the process, the cooling section of the plate heat exchanger uses coolant or cold water to bring the milk to 39.2^0 F (4^0 C).

3. **Ultrapasteurization.** Heating milk to 89 to 100^0 C (191 to 212^0 F) for 1 to 0.01 Second can extend the refrigerated shelf life of milk from 60 to 90 days.

Dairy Pasteurization Table -		
Temperature	**Time**	**Pasteurization Type**
62.8°C (145°F)	30 minutes	Vat Pasteurization
71.7°C (161°F)	15 seconds	High temperature short time Pasteurization (HTST)
89°C (191°F)	1.0 second	Ultra Pasteurization (UP)
90°C (194°F)	0.5 seconds	Ultra Pasteurization (UP)
94°C (201°F)	0.1 seconds	Ultra Pasteurization (UP)
96°C (204°F)	0.05 seconds	Ultra Pasteurization (UP)
100°C (212°F)	0.01 seconds	Ultra Pasteurization (UP)
138°C (280°F)	2.0 seconds	Ultra-high temperature (UHT) Sterilization

Phosphatase test (determination of the efficiency of pasteurization of milk)

Aim: - To determine the efficiency of pasteurization of milk by phosphatase test.

Theory: -

The test is based on the presence of heat labile enzyme phosphatase present in the raw milk and also produced by several pathogenic bacteria. The test determines the inactivation of enzyme phosphatase. This enzyme is capable of liberating phosphate from wild variety of substrates like di-sodium phenyl phosphate, glycerol phosphate. In this test the substrate used is di-sodium salt of para-nitro phenyl phosphate. The enzyme phosphatase is capable of degrading this substrate and liberating **para-nitro phenol which is a yellow** coloured compound.

Phosphatase is an enzyme which is destroyed at pasteurization temperature. That is at 62.8 ^{0} C for 30 minutes or 71.6 ^{0} C for 15 seconds. Thus the absence of phosphatase indicates efficient pasteurization and the development of yellow colour due to presence of phosphatase indicates inefficient pasteurization.

Requirements: -

1. Buffer substrate solution (di-sodium para-nitro phenyl phosphate in buffer)
   Buffer solution (3.5 gm of Na_2CO_3 + 1.5 gm $NaHCO_3$ + 1 litre D/W).

   0.15 gm di-sodium para-nitro phenyl phosphate in 100 ml of above buffer solution.
2. Test tubes 10 ml capacity 3
3. Pipettes
4. Incubator adjusted at 37 ^{0}C
5. Milk sample

Procedure: -

1. **Test: -** 5 ml of buffer substrate is transferred to a test tube. 1 ml of milk sample is added to it. The contents are shaken well to mix.
2. **Positive control : -** 5 ml raw milk

3. **Negative control: -** 5 ml of buffer substrate plus 1 ml boiled milk
4. All the tube are incubated at 37^0 C for 2 hours and observed for yellow colour formation.

Observation: -

If Yellow colour is observed, Pasteurization is not done properly. If yellow colour is not observed, pasteurization is done properly.

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* Sterilization of milk / Ultra-high temperature (UHT) processing /or Aseptic processing

While pasteurization conditions effectively eliminate potential pathogenic microorganisms, it is not sufficient to inactivate the thermoresistant spores in milk. The term **sterilization** refers to the complete elimination of all microorganisms. The food industry uses the more realistic term **"commercial sterilization"**; a product is not necessarily free of all microorganisms, but those that survive the sterilization process are unlikely to grow during storage and cause product spoilage.

The most recent method allows milk to be treated at 138^0 C (280^0 F) to 141°C (286°F) for two seconds; followed by packaging in airtight containers allows storage without refrigeration for up to 90 days.

Milk can be made commercially sterile by subjecting it to temperatures in excess of 100° C, and packaging it in air-tight containers. The milk may be packaged either before or after sterilization. The basis of **UHT**, or ultra-high temperature, is the sterilization of food **before** packaging, then filling into pre-sterilized containers in a sterile atmosphere. Milk that is processed in this way using temperatures exceeding 135° C, permits a decrease in the necessary holding time to 2-5 seconds) enabling a **continuous flow** operation.

There are two principal methods of UHT treatment:

A. Direct Heating
B. Indirect Heating

A) Direct heating systems

The product is heated by direct contact with steam of potable or culinary quality. The main advantage of direct heating is that the product is held at the

elevated temperature for a shorter period of time. For a heat-sensitive product such as milk, this means less damage.

There are two methods of direct heating

1. injection
2. infusion

1. Injection: High pressure steam is injected into pre-heated liquid by a steam injector leading to a rapid rise in temperature. After holding, the product is flash-cooled in a vacuum to remove water equivalent to amount of condensed steam used. This method allows fast heating and cooling, and volatile removal, but is only suitable for some products. It is energy intensive and because the product comes in contact with hot equipment, there is potential for flavour damage.

2. Infusion: The liquid product stream is pumped through a distributing nozzle into a chamber of high pressure steam. This system is characterized by a large steam volume and a small product volume, distributed in a large surface area of product. Product temperature is accurately controlled via pressure. Additional holding time may be accomplished through the use of plate or tubular heat exchangers, followed by flash cooling in vacuum chamber.

B) Indirect heating systems

The heating medium and product are not in direct contact, but separated by equipment contact surfaces. Several types of heat exchangers are applicable:

- plate
- tubular
- scraped surface

Packaging for Aseptic Processing

The most important point to remember is that it must be **sterile**! All handling of product post-process must be within the sterile environment.

There are 5 basic types of aseptic packaging lines:

1. **Fill and seal:** preformed containers made of thermoformed plastic, glass or metal are sterilized, filled in aseptic environment, and sealed
2. **Form, fill and seal:** roll of material is sterilized, formed in sterile environment, filled, sealed e.g. tetrapak

3. **Erect, fill and seal:** using knocked-down blanks, erected, sterilized, filled, sealed. e.g. gable-top cartons, cambri-bloc
4. **Thermoform, fill, sealed** roll stock sterilized, thermoformed, filled, sealed aseptically. e.g. creamers, plastic soup cans
5. **Blow mold, fill, seal:**

There are several different **package forms** that are used in aseptic UHT processing:

- cans
- paperboard/plastic/foil/plastic laminates
- flexible pouches
- thermoformed plastic containers
- flow molded containers
- bag-in-box
- bulk totes

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