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Qualitative analysis of milk samples and isolation, identification and characterization of micro-organisms present in the milk samples

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Abstract: Milk being rich in the protein casein is a significant food of nutrition for immense population on earth. It is the primary source of nutrition for young mammals and human infants. Some important nutrients like calcium, phosphorous, vitamin-B, potassium, vitamin-D and proteins especially casein. Milk supplement in daily diet makes the bones stronger and prevent them from fractures and other skeletal diseases. As milk is highly nutritious, it serves as a substrate for the growth of many micro-organisms especially bacteria like *Escherichia coli, Mycobacterium bovis, Salmonella sps, Listeria monocytogenes, Yersienia enterocolifica, Camphylobacter jejuni,* etc. the presence of these microbes in milk reduces the quality of milk and when consumed causes various diseases to the consumer. So it is highly necessary to ensure the quality of milk before consumption. Methylene Blue Reduction Test is usually performed for the qualitative analysis of milk. When the bacterial load in milk increases, methylene blue (indicator) gets reduced due to the decrease of oxidation-reduction potential of milk from 0.06V-0.01V. The organisms present in the milk samples were isolated, identified and characterized using staining techniques like Gram's staining and lacto phenol cotton blue staining and various biochemical tests like catalase test, oxidase test, coagulase test, urease test, citrate utilization test, MR-VP test, motility test and carbohydrate fermentation test were also performed. *Staphylococcus sps, Streptococcus sps, Klebsiella sps and Lactobacillus sps* were tested for sensitivity reactions against various antibiotics like erythromycin, glindamycin, gentamycin, ciprofloxacin, amoxicillin, penicillin, cephalosporin and streptomycin.

Keywords: Milk quality, MBRT, milk spoiling microbes, milk spoilage.

I. INTRODUCTION:

Milk is a nutrient-rich liquid and it is the lacteal secretion obtained by the complete milking of mammalian animals. Due to its high nutritional value for human beings, it is a significant food of nutrition of immense population on earth. It is the primary source for young mammals, source of nutrition for young mammals, including breastfed human infants before they are able to digest solid food. Early- lactation milk is called colostrum, which contains antibodies that strengthen the immune system and thus reduces the risk of many diseases. Interspecies consumption of milk is not uncommon, particularly among humans, many of whom consume the milk of other mammals. It is packed with important nutrients like calcium, phosphorus, B vitamins, potassium, and vitamin D, plus, it's an excellent source of protein. Drinking milk and dairy products may prevent osteoporosis and bone fractures and even help you maintain a healthy weight. For the expectant lactating mothers as well as for growing children milk is an important part of daily diet. Air, soil, feed, milking equipment, grass, and feces are the different sources of microbial contamination of raw milk.

Milk is a highly nutritious growth medium for microorganisms as it is rich in carbohydrates, fats, case in, protein, vitamins, and minerals. When temperature is suitable for growth of microorganism, the milk appears as an excellent medium for their growth. The milk is contaminated very easily if it is handled carelessly and produced un-hygienically results in its early spoilage. Due to its high moisture content and nutrition, it serves as a great medium for growth of microorganisms. Those microorganisms are pathogenic to human beings. Quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplications of many kind of microorganisms. The contamination of milk and milk products is largely due to human factor and unhygienic conditions. Usually milk gets contaminated with different kinds of microorganisms at milk collecting places. Milk is a major part of human food and plays a prominent role in the diet. Approximately 50% of milk produced is consumed as fresh or boiled, one sixth as yogurt or curd and remaining is utilized for manufacturing of indigenous varieties of milk products such as ice cream, butter, khoa, paneer, rabir, kheer, burfi, and gulabjaman. The manufacture of these products is based on traditional method without any regard to the quality of the raw material or the hygiene quality of the products. Under such conditions many microorganisms can find access to the milk products. Mostly food-borne diseases are among main public health disquiet throughout the world. Raw and pasteurized milk are daily consumed by millions of people. As a result infected milk either during milk processing or from infected cow results in different zoonotic diseases to many of them. The diseases include brucellosis, typhoid fever and salmonella food poisoning, tuberculosis, gastroenteritis, Q- fever, dysentery, diphtheria, and staphylococcal intoxications. Pasteurized milk is raw milk that has been heated to a specific temperature and time to kill pathogens that may be found in in the raw milk. Pathogens are microorganisms such as bacteria that make us sick. Raw milk can contain pathogens and other bacteria. Raw milk includes milk from cows, goats, sheep, and other dairy animals. By law, all milk sold to the public must us be pasteurized and packaged in a licensed dairy plant. Only vitamins A and D may be added to the milk, no other additives or preservatives can be legally added milk. Vitamin A improves eyesight, helps you to see better at night or in dim light, and helps you to tell colours apart. Vitamin D helps your body absorb calcium.

Milk testing and quality control is an essential component of any milk processing industry whether small, medium or large scale. Milk being made up of 87% Water prone to adulteration by unscrupulous middlemen and unfaithful farm workers. Moreover, its high nutritive value makes it an ideal medium its high nutritive value makes it an ideal deal medium for the rapid multiplication of bacteria, particularly under unhygienic production and storage at ambient temperatures. We know that, in order for any processor or handler will only be assured for the raw milk if certain basic quality tests are carried out at various stages of transportation of milk from the producer to the processor and finally to the consumer. The raw milk and pasteurized milk gives growth to microorganism that might get varies slightly. Not all the milk pathogens can able to grown in the pasteurized milk. Microorganisms in the milk are capable of causing infection in such cases, identifying the microorganism in the milk and about their pathogenicity is the most important work. If, we know all the microorganisms in the milk and their pathogenicity we could be able to manage the problems regarding body health and we could take all precautions. So, we could able to manage the problems.

Present study was carried out to determine the microorganisms present in different milk samples. Microorganisms plays an important role in dairy products. Microorganisms plays an important role in dairy products. Microorganisms are capable of doing wonderful magic in dairy products and cause serious illness, hospitalization even death.

1. MILK COMPOSITION:

Milk contains more water than any other element, around 87% for dairy cows. The other elements are dissolved, colloidally dispersed, and emulsified in water.

The quantities of the main milk constituents can vary considerably depending on the individual animal, its breed, stage of lactation, age and health status. Herd management practices and environmental conditions also influence milk composition. The average composition of cow's milk is shown in Table

1.1 COMPOSITION OF COW'S MILK:

Main constituent	Range (%)	Mean (%)
Water	85.5-89.5	87.0
Total solids	10.5-14.5	13.0
Fat	2.5-6.6	4.0
Proteins	2.9-5.0	3.4
Lactose	3.6-5.5	4.8
Minerals	0.6-0.9	0.8

1.1.1 MILK FAT

If milk is left to stand, a layer of cream forms on the surface. The cream differs considerably in appearance from the lower layer of skim milk. Under the microscope cream can be seen to consist of a large number of spheres of varying sizes floating in the milk. Each sphere is surrounded by a thin skin (the fat globule membrane) which acts as the emulsifying agent for the fat suspended in milk. The membrane protects the fat against enzymes and prevents the globules coalescing into butter grains. The fat is present as an oil-in-water emulsion: this emulsion can be broken by mechanical action such as shaking.

About 98% of milk fat is a mixture of triacylglycerols, with much smaller amounts of free fatty acids, mono-and diacylglycerols, phospholipids, sterols, and hydrocarbons. Milk fat also contains pigments (e.g. carotene, which gives butter its yellow colour), and waxes. Milk fat acts as a solvent for the fat-soluble vitamins A, D, E and K and also supplies essential fatty acids (linoleic, linolenic and arachidonic).

Proteins perform a variety of functions in living organisms ranging from providing structure to reproduction. Milk proteins represent one of the greatest contributions of milk to human nutrition. Proteins are polymers of amino acids. Only 20 different amino acids occur, regularly in proteins.

The content and sequence of amino acids in a protein therefore affect its properties. Some proteins contain substances other than amino acids, e.g. lipoproteins contain fat and protein. Such proteins are called conjugated proteins as phosphoproteins, lipoproteins and chromoproteins. The phosphate phosphate is linked chemically to phosphoproteins, the casein in milk is an example. A combination of lipid and protein forms the lipoprotein and are excellent emulsifying agents. Chromoproteins are proteins with a coloured prosthetic group and include haemoglobin and myoglobin.

1.1.2 MILK PROTEINS

a. CASEIN

The casein is a group name for the dominant class of protein in milk. Normal bovine milk contains about 3.5% protein, ok which casein constitutes about 80%. Casein is easily separated from milk, either by acid precipitation or by adding rennin. In cheesemaking most of the casein is recovered with the milk fat. Casein can also be recovered from skim milk as a separate product. Casein is dispersed in milk in the form of micelles. The micelles are stabilised by the K-casein. Caseins are hydrophobic but K-casein contains a hydrophilic portion known as the glycomacropeptide and it is this that stabilises the micelles. The structure of the micelles is not fully understood. When the pH of milk is changed, the acidic or basic groups of the proteins will be neutralised. At the pH at which the positive charge on a protein equals exactly the negative charge, the net total charge of the protein is zero. This pH is called the isoelectric point of the protein (pH 4.6 for casein). If an acid is added to milk, or if acid-producing bacteria are allowed to grow in milk, the pH falls. As the pH falls the charge on casein falls and it precipitates. Hence milk curdles as it sours, or the casein precipitates more completely at low pH.

b. WHEY PROTEINS

The whey proteins are also made up of a number of distinct proteins as shown in the scheme. Whey protein comprises the group of proteins in whey during the cheesemaking process. Whey protein also contains fragments of casein molecules. After the fat and casein have been removed from milk, one is left with whey, which contains the soluble milk salts, milk sugar and the remainder of the milk proteins. Like the proteins in eggs, whey proteins can be coagulated by heat. When coagulated, they can be recovered with caseins in the manufacture of acid-type cheeses. The whey proteins are made up of a number of distinct proteins, the most important of which are b-lactoglobulin and lactoglobulin. b-lactoglobulin accounts for about 50% of the whey proteins, and has a high content of essential amino acids. It forms a complex with K-casein when milk is heated to more than 75°C, and this complex affects the functional properties of milk. Denaturation of b-lactoglobulin causes the cooked flavour of heated milk.

c. OTHER MILK PROTEINS

In addition to the major protein fractions outlined, milk contains a number of enzymes. The main enzymes present are lipases, which cause rancidity, particularly in homogenized milk, and phosphatase enzymes, which catalyze the hydrolysis of organic phosphates. Measuring the inactivation of alkaline phosphatase is a method of testing the effectiveness of pasteurization of milk. Peroxidase enzymes, which catalyze the breakdown of hydrogen peroxide to water and oxygen, are also present. Lactoperoxidase can be activated and use is made of this for milk preservation. Milk also contains protease enzymes, which catalyze the hydrolysis of proteins, and lactalbumin, bovine serum albumin, the immune globulins and lactoferrin, which protect the young calf against infection.

1.1.3 MILK CARBOHYDRATES

Lactose is the major carbohydrate fraction in milk. It is made up of two sugars, glucose and galactose. The average lactose content of milk varies between 4.7 and 4.9%, though milk. Lactose is a source of energy for the young calf, and provides 4 calories/g of lactose metabolized. It is less soluble in water than sucrose and is also less sweet. It can be broken down to glucose and galactose by bacteria that have the enzyme b-galactosidase. The glucose and galactose can then be fermented to lactic acid. This occurs when milk goes sour. Under controlled conditions they can also be fermented to other acids to give a desired flavour, such as propionic acid fermentation in Swiss-cheese manufacture. Lactose is present in milk as molecular solution. In cheese making lactose remains in the whey fraction. It has been recovered from whey for use in the pharmaceutical industry, where its low solubility in water makes it suitable for coating tablets. It is used to fortify baby-food formula. Lactose can be sprayed on silage to increase the rate of acid development in silage fermentation. It can be converted into ethanol using certain strains of yeast, and the yeast biomass recovered and used as animal feed. However, these processes are expensive and a large throughput is necessary for them to be profitable. For smallholders, whey is best used as a food without any further processing.

Heating milk to above 100oC causes lactose to combine irreversibly with the milk proteins. This reduces the nutritional value of the milk and also turns it brown. Because lactose is not as soluble in water as sucrose, adding sucrose to milk forces lactose out of solution and it crystallizes. This causes sandiness in such products as ice cream. Special processing is required to crystallize lactose when manufacturing products such as instant skim milk powders. Some people are unable to metabolize lactose and suffer from an allergy as a result. Pre-treatment of milk with lactase enzyme breaks down the lactose and helps overcome this difficulty.

In addition to lactose, milk contains traces of glucose and galactose. Carbohydrates are also present in association with protein. K-casein, which stabilizes the casein system, is a carbohydrate-containing protein.

1.1.4 MILK SALTS

Milk salts are mainly chlorides, phosphates and citrates of sodium, calcium and magnesium. Although salts comprise less than 1 % of the milk they influence its rate of coagulation and other functional properties. Some salts are present in true solution. The physical state of other salts is not fully understood. Calcium, magnesium, phosphorous and citrate are distributed between the soluble and colloidal phases (Table 2). Their equilibria are altered by heating, cooling and by a change in pH.

In addition to the major salts, milk also contains trace elements. Some elements come to the milk from feeds, but milking utensils and equipment are important sources of such elements as copper, iron, nickel and zinc.

Table 2. Distribution of milk salts between the soluble and colloidal phases.

	Total	Dissolved	Colloidal
	(mg/100 ml of milk)		
Calcium	1320.1	51.8	80.3
Magnesium	10.8	7.9	2.9
Total phosphrous	95.8	36.3	59.6
Citrate	156.6	141.6	15.0

1.1.5 MILK VITAMINS

Milk contains the fat-soluble vitamins A, D, E and K in association with the fat fraction and water-soluble vitamins B complex and C in association with the water phase. Vitamins are unstable and processing can therefore reduce the effective vitamin content of milk their consumption can add diversity to plant-based diets. Animal milk can play an important role in the diets of children in populations with very low fat intakes and limited access to other animal source foods.

2. MICROORGANISMS OF CONCERN IN MILK

2.1 MOST COMMON MICRO-ORGANISMS OF CONCERN:

The milkborne pathogens and their associated illnesses, and several other important microorganisms in milk. A table summarizing the disease characteristics of the major milk pathogens is presented at the beginning of this section, followed by a more detailed discussion of the microorganisms in milk, listed in alphabetical order: *Brucella spp., Campylobacter jejuni, Coliforms, Coxiella burnetii, Escherichia coli O157:H7, Listeria monocytogenes, Mycobacterium bovis and tuberculosis, Mycobacterium paratuberculosis, Psychrotrophic Bacteria, Salmonella spp., and Yersinia enterocolitica*.

Cases of human illness associated with the consumption of dairy products are listed in the Disease Outbreaks Associated with Milk Products Section.

Major milk borne diseases pathogens and their associated diseases:

Organism	Diseases	Diseases symptoms	Source
Camphylobacter jejuni	Gastroenteritis	Diarrhea, fever, abdominal pain	Intestinal tract and feces
Coxiella burnetii	Q fever	Chills, fever, weakness, head ache, possible endocarditis	Infected cattle, sheep, and goats
E. coli	Gastroenteritis, hemolytic uremic syndrome(HUS)	Diarrhea, abdominal pain, bloody diarrhea, kidney failure, possible death	Intestinal tract, and feces
listeria monocytogenes	Listeriosis	Flu-like symptoms, miscarriage, stillbirths, fetal death, and spontaneous abortion	Water, soil, environment
Mycobacterium paratuberculosis	Johne's (ruminants)	Unconfirmed link to Crohn's disease in humans	Infected animals
Mycobacterium bovis or paratuberculosis	Tuberculosis	Lung disease	Infected animals
Salmonella spp.	Gastroenteritis Typhoid fever	Diarrhea, nausea, fever	Feces and environment
Yersinia entrocolitica	Gastroentritis	Diarrhea , appendictis	Environment, water, infected animals

a. Brucella spp.

Brucella species (spp.) are found in many animal species including cattle, sheep, and goats. *Brucella spp*. are destroyed by pasteurization. *Brucella spp*. cause illness with symptoms that are flu-like and include fever, sweats, headaches, back pain and physical weakness. In some cases long-lasting symptoms of fever, joint pain and fatigue may occur.

b. Campylobacter jejuni

Campylobacter jejuni is found in the intestinal tract, udder, and feces of cattle, in poultry and wild birds, and in contaminated water sources. *Campylobacter jejuni* is destroyed by pasteurization. *Campylobacter jejuni* is one of the most common bacterial causes of diarrheal illness in the US. Illness can often occur as sporadic events and in larger outbreaks. *Campylobacter jejuni* generally causes illness 2 to 5 days after exposure, and illness typically lasts 5 to 10 days. Symptoms of campylobacteriosis include diarrhea, bloody diarrhea, abdominal pain, cramping, nausea, vomiting, and fever. Patients with Campylobacteriosis usually recover without specific treatment other than fluid and electrolyte replacement. In some persons with a compromised immune system, *Campylobacter jejuni* infection can lead to the more serious diseases Guillan-Barré syndrome and Reiter syndrome. Guillan-Barré syndrome is a disorder that results in temporary neuromuscular paralysis, although 20% of those infected may have long term disability and it may cause death. Reiter syndrome is a reactive arthritis that may affect multiple joints, particularly the knee joint. The prevalence of *Campylobacter jejuni* is very widespread. It has been reported in bulk tank raw milk samples in Illinois, Michigan, Minnesota, Ohio, Pennsylvania, South Dakota, Tennessee, Virginia, and Wisconsin, suggesting that the organism is ubiquitous. In these studies, Campylobacter jejuni was found in 0.4 to 12.3% of the bulk tank milk samples.

c. Coxiella burnetii

Coxiella burnetii is found in many animals worldwide and is shed in the milk, urine and feces of cattle, goats, and sheep. *Coxiella burnetii* is considered to be the most heat resistant non-sporeforming pathogen commonly found in milk, and the established conditions for milk pasteurization are specifically designed to destroy this organism. *Coxiella burnetii* causes Q fever, an illness characterized by a sudden onset of high fever, severe headache, nausea, vomiting, diarrhea, abdominal pain, chest pain, chills, sweats, sore throat, non-productive cough, and general malaise. Fever can last for 1 to 2 weeks. Most patients recover without any treatment, although *Coxiella burnetii* may result in death. The prevalence of Coxiella burnetii was >94% in raw milk samples from the Northeastern, Midwestern, and Western regions of the US tested between 2001 and 2003.

d. Escherichia coli O157:H7

Escherichia coli O157:H7 is one strain in a large family of bacteria. Strains of Escherichia coli (E. coli) are considered fecal coliforms. Most strains of Escherichia coli do not cause illness and live in the intestinal tracts of healthy humans and animals. *E. coli O157:H7* is found in the intestinal tract and feces of cattle. *E. coli O157:H7* is destroyed by pasteurization. *E. coli O157:H7* produces toxins that cause illness in humans. Symptoms of illness include bloody diarrhea and abdominal cramps. In some cases, particularly in young children, *E. coli* O157:H7 infection causes hemolytic uremic syndrome, which destroys red blood cells and causes kidney damage or failure, and in some cases death. The prevalence of *E. coli* O157:H7 and Shiga-toxin producing E. coli has been reported for bulk tank raw milk samples in Minnesota, Pennsylvania, South Dakota, Wisconsin and Ontario. *E. coli* O157:H7 was found in 0.87 to 10% of the bulk tank milk samples tested.

e. Listeria monocytogenes

Listeria monocytogenes is found in soil and water and has been isolated from a large number of environmental sources. *Listeria monocytogenes* is destroyed by pasteurization, but if food products are contaminated after pasteurization, *Listeria monocytogenes* can grow at refrigerator temperatures. Illness can occur as sporadic events or larger outbreaks. *Listeria monocytogenes* typically causes illness in pregnant adults, newborns, the elderly, and patients with compromised immune systems, but healthy adults and children may also become infected. Symptoms of Listeriosis include flu-like symptoms, fever, muscle aches, stiff neck, headache, septicemia, meningitis, miscarriage, stillbirth, premature delivery, abortion, or death. The prevalence of *Listeria monocytogenes* has been reported for bulk tank raw milk samples in individual states (or grouped by region) for California, Colorado, Florida, Idaho, Illinois, Indiana, Iowa, Kentucky, Massachusetts, Michigan, Minnesota, Missouri, Nebraska, New Mexico, New York, Ohio, Pennsylvania, South Dakota, Tennessee, Texas, Washington, Wisconsin, Vermont, Virginia, and in Alberta and Ontario, Canada. *Listeria monocytogenes* was found in up to 12% of the bulk tank milk samples tested, illustrating the widespread presence of *Listeria monocytogenes* in unpasteurized milk.

f. Mycobacterium bovis

Mycobacterium bovis and *Mycobacterium tuberculosis* are found in infected cattle worldwide. Both of these organisms are destroyed by pasteurization. *Mycobacterium bovis* and *Mycobacterium tuberculosis* cause tuberculosis, a lung disease. Tuberculosis in the US is not very common today, although historically milk was a common source of tuberculosis. Tuberculosis is a concern in many parts of the world.

2.2 OTHER BACTERIA OF SIGNIFICANCE:

2.2.1 COLIFORMS:

Coliforms are a large group of bacteria that are found in the intestines of warm-blooded animals. Most coliforms are not pathogenic, but their presence indicates contamination, usually from fecal sources. Coliforms are destroyed by pasteurization. The prevalence of coliforms were detected in 62 to 95% of the raw bulk tank milk tested in regions that included California, Colorado, Florida, Idaho, Illinois, Indiana, Iowa, Kentucky, Michigan, Minnesota, Missouri, New Mexico, New York, Ohio, Pennsylvania, South Dakota, Tennessee, Texas, Washington, Wisconsin, Vermont, and Virginia, suggesting the ubiquitous presence of these organisms in unpasteurized milk.

2.2.2 PSYCHROTROPHIC BACTERIA

Psychrotrophic bacteria are not a specific type or family of bacteria, but rather this is the name given to bacteria that are capable of growing at 44.6°F (7°C) or less. This group of microbes is a concern in dairy products because they grow at refrigerator temperature and cause spoilage, often resulting in off-flavors. The most common psychrotrophs are in the genus Pseudomonas. These organisms are killed by pasteurization, but may occur in milk from contamination after pasteurization. Some bacterial pathogens are psychrotrophic, including *Listeria monocytogenes*, *Yersinia enterocolitica*, some *E. coli* strains and some Bacillus strains.

a. Salmonella sp

Salmonella are among the main causes of food poisoning. The World Health Organisation (WHO) expects the number of worldwide infections to total around 16 million each year, more than half a million of them being fatal. Salmonella are killed by pasteurization; raw milk and dried milk products are vulnerable, though.

b. E. coli

Certain species of the bacterium *E. coli* produce shigatoxin which may cause symptoms like diarrhea and stomach pain, sometimes even leading to life-threatening complications like hemolytic-uremic syndrome. Although *E. coli* are killed by pasteurization, infections due to milk products occur time and again. Last year, Germany was affected by a major recall of long-life milk, and recently, infected goat cheese was recalled in France.

c. Pseudomonas sp

Pseudomonads are opportunistic pathogens. The species *Pseudomonas fluorescens* is among the most common spoilage agents in milk. The enzymes produced by pseudomonads are heat-stable and can survive pasteurization.

d. Bacillus cereus

The bacterium Bacillus cereus causes spoilage of cream, cheese and milk and makes them taste rancid, sour or bitter. In addition, it produces toxins which may cause serious gastrointestinal disorders. In February this year, a discounter in Germany had to recall long-life whole milk due to Bacillus cereus contamination. Strict adherence to the cold chain is essential to prevent the bacteria from multiplying. Spores of Bacillus cereus can survive heat treatment.

e. Clostridia sp

Clostridia form heat-resistant spores which can survive pasteurization at least partially. The species *Clostridium tyrobutyricum* is particularly feared in dairy production, because it causes so-called late blowing in raw milk cheese. Affected cheese is no longer saleable. Clostridia usually get into the milk via infected feed, hay or feces.

3. MBRT

Methylene Blue Reduction Test also known as mbrt test. It is a qualitative test for milk, it used to check the quality of raw and pasteurized milk. The Methylene Blue Reduction Test is based on the fact that in the presence of oxygen the methylene blue solution forms blue color, and it will lose the color as the oxygen is depleted. The bacteria present in the milk will ferment lactose (milk sugar) to form lactic acid, during this fermentation process the oxygen is used up, which causes in depletion of oxygen in milk, and electrons are released. These electrons react with the methylene blue solution. As a result, it decolorizes the methylene blue. Mainly bacteria are responsible for the oxygen consumption in milk. It is estimated that assumed that the greater the number of bacteria in milk, the quicker will the oxygen be consumed. The total number of microorganisms in milk. This test is performed in a dairy reception dock, processing units, and milk chilling centers. There this process is followed as acceptance/rejection criteria for the raw and processed milk.

3.1 AIM OF METHYLENE BLUE REDUCTION TEST

This test is performed to check the bacteria contamination in milk. It will visually indicate the presence of bacteria in a given milk sample, and it will indicate the level of milk quality.

3.2 METHYLENE BLUE REDUCTION TEST PRINCIPLE

Milk has sufficiently low redox potential which reduces the methylene blue immediately. During the milking, cooling, dumping the oxidation-reduction potential of milk is increased to +0.3V. At this point, the methylene blue remains in an oxidized state. When the bacterial cells are started to increase their numbers in milk it consumes more dissolved oxygen from the milk, as a result, the oxygen gets depleted. Then the Methylene Blue starts acting as an electron acceptor instead of oxygen. The methylene blue gets reduced due to the decreases of oxidation-reduction potential from + 0.06 to 0.01 V. The double-bonded nitrogen atom of Methylene Blue dye accepts 1 atom of hydrogen as a result the dye is converted into a colorless state. The greater is the number of microorganisms in milk, the greater is the metabolic activity and the faster is the reduction of methylene blue.

3.3 MATERIALS

- Methylene blue solution, 1% aqueous
- Milk sample
- Test tube
- Test tube stopper
- Pipet
- Water bath

3.4 METHYLENE BLUE REDUCTION TEST PROCEDURE

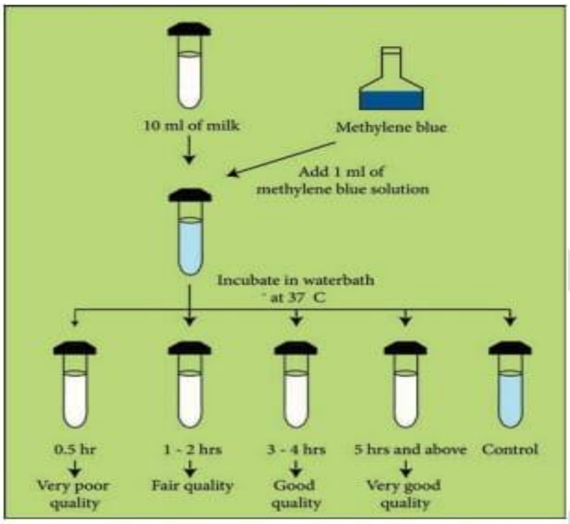
- Mix the milk sample thoroughly to distribute the fat uniformly.
- Add 10ml of milk sample in a test tube.
- Then add 1ml of standard methylene blue solution in this test tube and invert the test tube to mix it properly.

- After that, place the test tube in a water bath at 37°C (99°F) for 30 minutes, and Cover the bath with a lid.
- After 30 minutes of incubation observe the sample and check for discoloration, and make subsequent readings at hourly intervals thereafter.
- After each reading, remove decolorized tubes and then slowly make one complete inversion of remaining tubes.
- Record reduction time in whole hours between last inversion and decolorization. For example, if the sample were still blue after L 5 hours but was decolorized (white) at the 2.5-hour reading, the methylene blue reduction time would be recorded as 2 hours. Decolorization is considered complete when four-fifths of the color has disappeared.

3.5 INTERPRETATION

POSITIVE RESULT: If the viable bacteria decolorize the milk within 30 minutes, the milk is considered unsatisfactory.

NEGATIVE RESULT: If the milk is not decolorized within 30 minutes, the milk is considered as good quality.



Methylene Blue dye Reduction Test

II. MATERIALS AND METHODS

1. COLLECTION OF SAMPLES

All samples of milk were collected from surrounding areas of Coimbatore. The packet milk samples were also collected from the grocery shops. The milk sample were collected using the sterile sample bottles. The samples were taken to the laboratory and kept in the refrigerator

2. ISOLATION OF MICROORGANISMS:

Serial dilution was done to get minimized bacterial colonies for isolation and identification. 1 ml of the serially diluted milk samples were pipetted into each serially marked petri dish. The total microbial count was carried on the milk sample using pour plate method. Nutrient agar and potato dextrose agar were used for bacteria and fungi respectively. The plates were subsequently incubated at the 37°C for 24 hours for bacteria and 72 hours for fungi. At the end of incubation, developed colonies were counted and colonies forming unit of samples were calculated and recorded.

The isolated bacterial colonies were streaked on the MacConkey agar for observation of lactose fermenting microorganisms.

3. CHARACTERIZATION AND IDENTIFICATION ISOLATES:

Discrete colonies that developed after incubation, were sub-cultured to obtain pure culture and subsequently used for microscopic characterization and biochemical analyses. The plates were observed for the morphological and cultural characterization including the nature of margin, elevation, shape, colour and transparency. The isolates were further characterized and identified by biochemical procedures.

LPCB and gram staining were used for staining fungi and bacteria respectively

4. MBRT:

The methylene blue reduction test is based on the fact that the color imparted to milk by the addition of a dye such as methylene blue will disappear more or less quickly. The removal of the oxygen from milk and the formation of reducing substance during bacterial metabolism cause the colour to disappear. The oxygen is consumed by the bacteria. The greater the number of bacteria in milk, quicker will the oxygen be consumed, and in turn the sooner will the colour disappear.

5. ANTIBIOTIC SENSITIVITY TESTING:

The standardized disc diffusion method and the zone size interpretation chart were used for the determination of the bacterial sensitivity to the various antibiotics selected.

The following commercially prepared paper discs impregnated with the various antibiotics were assessed against the isolates; erythromycin, clindamycin, gentamycin, ciprofloxacin, chloramphenicol, amoxicillin, penicillin, cephalosporin, streptomycin, amphotericin B.

The paper discs were firmly placed on the inoculated plates. The plates were incubated at 37℃ for 24hours after which zone of inhibition were measured and recorded.

III. RESULTS:

1. CHARACTERIZATION AND ISOLATION OF MICROORGANISM

S.no	Sample	Microorganisms	Lactose fermentation
1	Cow cattle 1	*Staphylococcus sp, Streptococcus sp, Klebsiella sp, Lactobacillus* sp	*Klebsiella sp, Lactobacillus* sp
2	Milk booth	*Staphylococcus sp, Streptococcus sp, Pseudomonas sp, Lactobacillus* sp	*Lactobacillus* sp
3	Packet milk 1	*Staphylococcus sp, Aspergilus sp, Pseudomonas sp*	-
4	Cow cattle 2	*Staphylococcus sp, Streptococcus sp, Klebsiella sp, Lactobacillus* sp	*Klebsiella sp, Lactobacillus* sp
5	Packet milk 2	*Staphylococcus sp, Aspergilus sp, Pseudomonas sp*	-

S.no	Isolate	Gram staining reaction	Catalase	Oxidase	Coagulase	Citrate	Urease	MR	VP	Mobility	Lactose	Glucose	Sucrose
1	*Staphylococcus* sp	+	+	-	-	-	-	-	+	-	+	+	+
2	*Streptococcus* sp	+	-	-	-	+	-	+	-	-	+	+	+
3	*Klebsiella* sp	-	+	+	-	+	+	-	+	-	+	+	+
4	*Pseudomonas* sp	-	+	+	-	+	-	-	-	+	-	+	-
5	*Lactobacillus* sp	+	-	+	+	+	-	+	-	-	+	+	_-

S.no	Sample	LPCB	PDA	Organism
1	Packet milk 1	Septate hyphae, unbranched conidiophore	Black surface, texture velvety and cottony, reverse is brown	*Aspergillus* sp
2	Packet milk 2	Septate hyphae, unbranched conidiophore	Brown surface, texture velvety and cottony, reverse is golden	*Aspergillus* sp

2. MBRT:

S.no	Sample	Reduction time	Quality of milk
1	Cow cattle 1	2 hours	Bad
2	Milk booth	3 hours	Fair
3	Packet milk 1	5.30 hours	Good
4	Cow cattle 2	4 hours	Fair
5	Packet milk 2	6 hours	Good

3. ANTIBIOTIC SENSITIVITY TESTING:

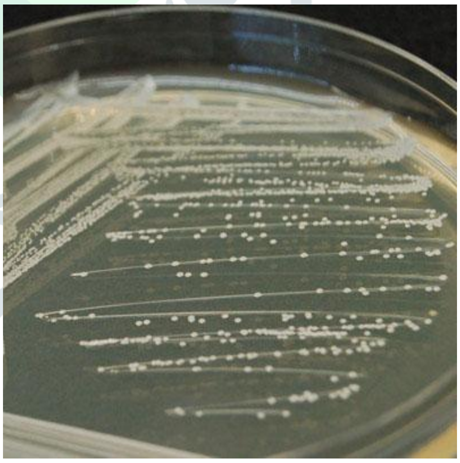
S.no	Discs	*Staphylococcus sp*	*Streptococcus sp*	*Pseudomonas sp*	*Klebsiella sp*	*Lactobacillus sp*
1	Erythromycin	13	23	NA	NA	18
2	Clindamycin	10	NA	NA	NA	12
3	Gentamycin	18	NA	20	18	10
5	Ciprofloxacin	NA	NA	16	28	NA
6	Amoxicillin	NA	20	NA	10	NA
7	Penicillin	8	24	NA	NA	22
8	Cephalosporin	NA	23	15	NA	NA
9	Streptomycin	NA	NA	18	NA	NA

Milk samples

Serial dilution

CULTURE PLATES:

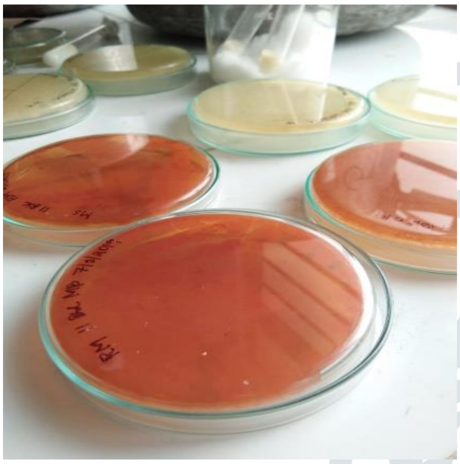
Nutrient agar plates



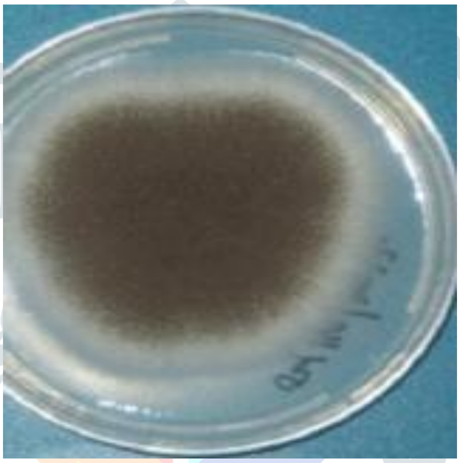
Klebsiella sp

Lactobacillus sp

Non Lactose fermenting colonies



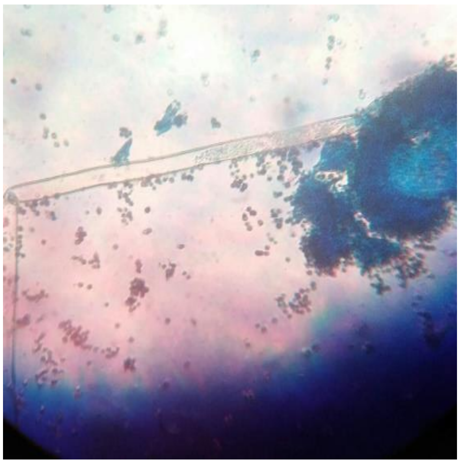
Mac Conkey agar plates



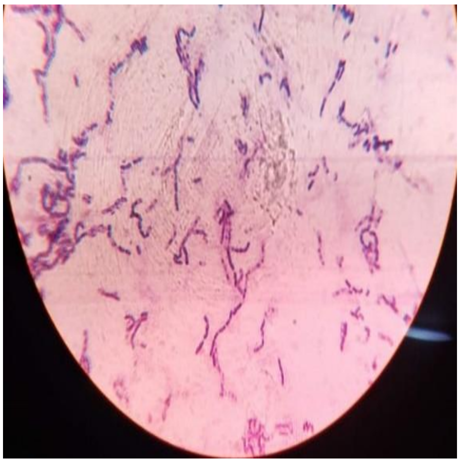
Aspergillus sp

Aspergillus sp

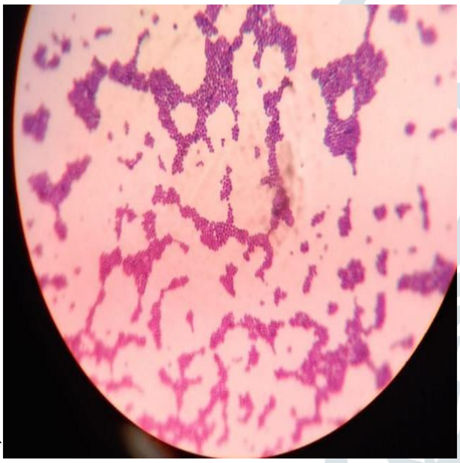
MICROSCOPIC OBSERVATION:



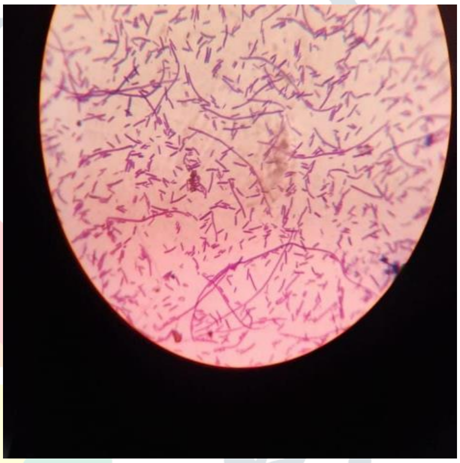
Aspergillus sp



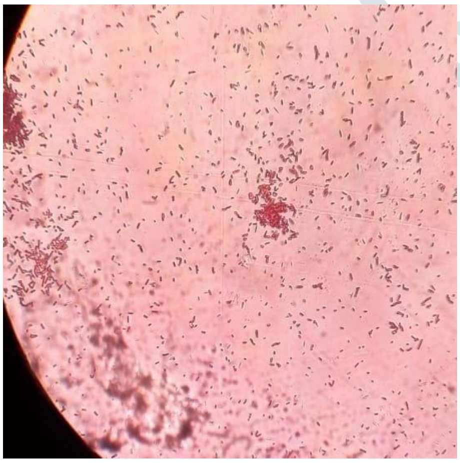
Pseudomonas s



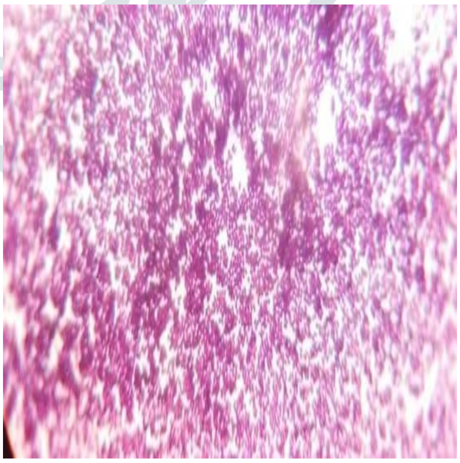
Staphylococcus sp



Lactobacillus sp

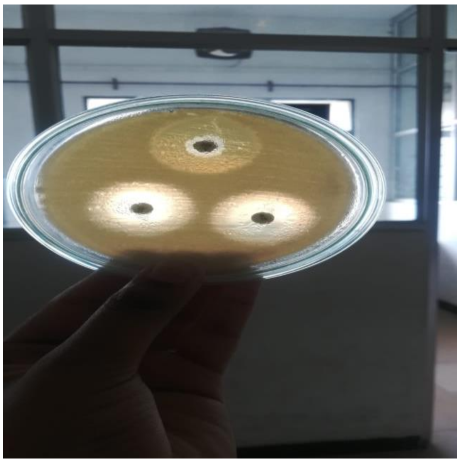


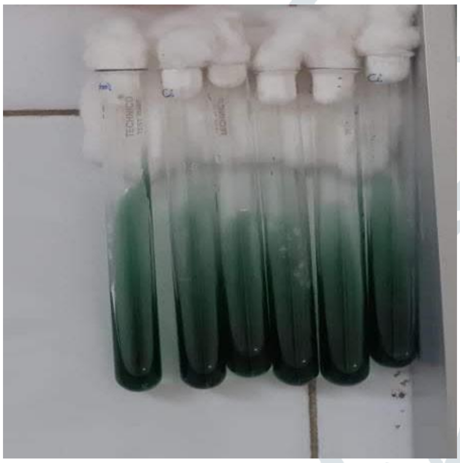
Klebsiella sp



Streptococcus sp

ANTIBIOTIC SENSITIVITY TEST:



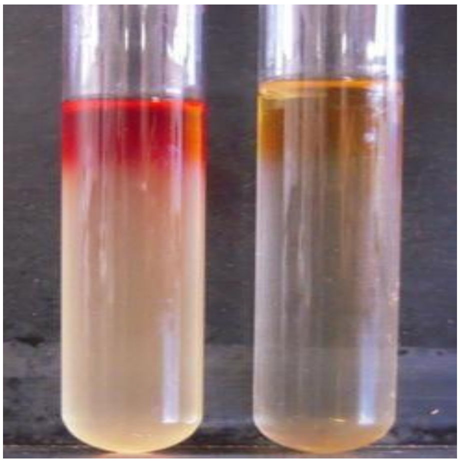


Oxidase test

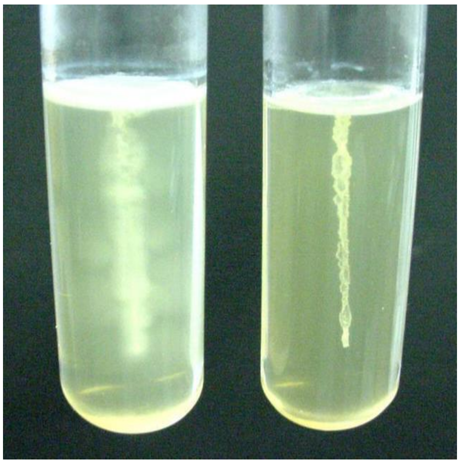
Catalase test

Citrate positive tubes

Citrate test



MR-VP test



Motility test

IV. DISCUSSION:

Milk is a valuable food resources that can meet the growing nutritional demand of the large population in the world. Microbial contamination in the milk poses a serious public health threat. The milk from different environment give rise to different microorganisms. This study revealed that pasteurized milk serves as a growth medium for *Aspergillus* sp.

The aerobic microorganisms during respiration O_2 serves as electron acceptor and hydrogen peroxide is formed in the cell. High concentration of hydrogen peroxide is toxic to the cell. Certain microbes posses the enzyme catalase that convert it into water and oxygen.

The aerobic microorganisms during respiration O_2 serves as electron acceptor and hydrogen peroxide is toxic to the cell. Certain microbes posses the enzymes catalase that convert it into water and oxygen. Aerobes mainly possess catalase enzymes. Aerobes do not. This is the reason why oxygen. This is the reason why oxygen is higly toxic to anaerobes (exception are there in broth).

In citrate utilization test, *Klebsiella* sp showed the colour change from green to Prussian blue is the plant which indicates positive result. Whereas *Staphylococcus* sp did not show colour change which indicate negative result.

Staphylococcus Sp does not have the ability to use citrate as the sole carbon and energy source but occasionally citrate utilizing

Oxidase is an enzyme possessed by some microorganism which favours the parts of electron transport system. It oxidase the reagent N-N-N- tetra methylene paraphenylene diamine dihydrochloride to a deep purple colored compound known as endophenol certain precautions have to be taken during experiment. The precautions are:

- Reagent have to be prepared fresh
- Nichrome wire should not be used to take the cultures as traces of iron gives positive result
- Colour change should be observed within prescriptional time

Urease, an enzyme possessed by bacterium hydrolysis. Urea is a diamide of carbonic acid. Urea acts upon urea splitting or hydrolysis it to ammonia and CO_2. Ammonia formed reacts in solution forming ammonia carbonate which is alkaline leading to the increase in Ph. Phenol red is an indicator incorporated in the medium which turned its colour from yellow to pink in an alkaline condition.

Proteus sp is an urease positive organism. The smell of open and uncleaned urinals is due to the presence of the presence of urea splitting organism which split urea realizing ammonia in environment can survive better in an acidic environment. Splitting urea and utilizing the ph.

Stuarts and christense urease. Agar are differential media used to detect urease activity in bacteria

Aspergillus sp is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans. In addition to its role as an opportunistic human pathogen.

The quality of packet milk is comparatively very good to consume.

In the dairy industry, the dye reduction test using methylene blue can determine whether or not milk is classified as “excellent”, “good”, “fair”, or “poor” based upon the rate at which the solution turns colorless (the faster the milk turns from blue to clear, the poorer the rating.) How does this work? Were your predictions about the milk samples correct? Why or why not?

When the bacterial cells are started to increase their numbers in milk it consumes more dissolved oxygen from the milk, as a result, the oxygen gets depleted. Then the Methylene Blue starts acting as an electron acceptor instead of oxygen. The methylene blue gets reduced due to the decreases of oxidation-reduction potential from + 0.06 to 0.01 V.The double-bonded nitrogen atom of Methylene Blue dye accepts 1 atom of hydrogen as a result the dye is converted into a colorless state. The greater is the number of microorganisms in milk, the greater is the metabolic activity and the faster is the reduction of methylene blue.

Methylene blue is used as a disinfectant and biological stain (NTP, 2008; Oz et al., 2011). As a disinfectant, methylene blue is sold to end-consumers as an aquarium fungicide (Schirmer et al., 2011).

V. CONCLUSION:

Different milk samples were collected, qualitatively analyzed and the micro-organisms present in them were isolated, identified and characterized using culturing, staining and biochemical tests and antibiotic sensitivity tests.

VI. ACKNOWLEDGMENT:

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