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Microbiological Analysis Of Raw And Pasteurized Milk By Qualitative Methods

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ABSTRACT

A sample of unpasteurized and pasteurized cow milk was collected to analyze and to compare the microbial growth and contamination. Both the samples were cultured in three different agars- Tryptone Glucose Yeast agar (TGYA), MacConkey agar, Eosin Methylene Blue agar (EMB) with the solutions diluted upto 10^{-3} and 10^{-4}. After incubating cultures at 30-35°C for 18-72 hours, bacterial growth was observed in agar with unpasteurized milk samples. Biochemical tests were also performed on the colonies formed on EMB agar that indicated the presence of pathogenic species. Methylene blue reduction test showed that pasteurized milk is of good quality as the reduction time was longer as compared to raw milk. The results obtained from this study demonstrated the unpasteurized milk is unsafe due high microbial contamination and why the process of pasteurization is crucial and therefore should not be neglected.

Keywords- Pasteurized, Contamination, EMB Agar, Culture, Incubation, Pathogenic

INTRODUCTION

Milk is secreted by mammals for the nourishment of their young ones. It is in liquid form without any colostrum. The milk contains water, fat, protein & lactose. About 80-85% of the proteins are casein proteins.

Microbiology of milk

High water content, moderate pH (6.4-6.6), good quantity of nutrients, etc. make milk an excellent medium for microbial growth. Milk always contains some bacteria derived from various sources -

A) Milk ducts of udder- Some bacteria are derived from udders. Their number is highest in foremilk & lowest in milk obtained by stripping. Aseptically drawn raw milk may contain between 500 to 1000 bacteria per ml.

B) Milking equipment- Unsterile milking equipment is a major contributor of bacteria in milk.

C) The milker - Milker's hands also contribute to bacteria in milk by improper cleaning of hands before milking the animals.

D) Water - Used for cleaning the udders & for adulteration, also partly contributes to bacteria in milk.

E) Diseased animals - Pathogenic organisms causing infection in animals (Ex. mastitis, brucellosis, and tuberculosis) may be excreted in milk.

Types of bacteria in milk: The various types of bacteria that may be encountered in milk are shown in the table 1:

Group	Organism	Sources
Acid forming	*Lactobacilli, Streptococcus*	Utensils
Alkali forming	*Alkaigenes supp., Achromobacter*	Environment
Gas forming	*Coliform bacilli, Clostridium wekhii, C. butyricum*	Environment
Proteolytic	*Bacillus subtilis, B. cereus, Proteus vulgaris*	Environment

Table-1 Bacteria present in milk

Pasteurization eliminates/ reduces the contamination of microbes and makes it safer for consumers. Pasteurization is a process in which packed milk is treated with mild heat, usually to less than 100°C, to

eliminate pathogens & extend shelf life. The process was named after the French scientist Louis Pasteur. It is intended to destroy or deactivate organisms & enzymes that contribute to spoilage or risk of disease.

Milk analysis can be done by various methods. The Methylene blue reduction and phosphatase test are methods widely used to detect the presence of microbes in pasteurized milk. The standard plate count is used to determine the total number of bacteria present in a specified amount of milk, usually a milliliter (mL).This can also be used for grading milk. The coliform plate count is typically used to determine the total number of coliforms present in 1 mL of milk sample.

3. OBJECTIVE

- To compare and study the microbial load in unpasteurized and pasteurized milk sample of Kota.
- To observe the growth of microorganisms on different agar media.
- To test the quality of milk samples collected from local vendor.
- To perform biochemical tests for identification of microorganisms present in milk samples.

4. MATERIALS AND METHODOLOGY

One pasteurized milk sample of a brand and one non-pasteurized raw milk sample from a local vendor were collected. The samples were stored in a refrigerator until used and were the serially diluted using phosphate buffered saline and labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. Samples diluted to $10^{-3}$and 10^{-4} were used. The sample was cultured in three agars- Tryptone Glucose Yeast Agar, MacConkey Agar and Eosin Methylene Blue Agar. Tryptone Glucose Yeast Agar is used for counting bacteria in water and dairy products. MacConkey Agar is a culture medium used for isolation, differentiation and cultivation of coliform organisms. Eosin Methylene Blue Agar (EMB) is a selective for gram-negative bacteria and provides a color indicator distinguishing between organisms that ferment lactose and those that do not. One (1) mL of each sample was then added to approximately 20 mL of agar in petri plates. The plates were incubated at 37^0 C for 24 hours.

Gram Staining

The standard protocol for gram staining was performed on the colonies. The slide was then viewed under the oil immersion objective (×100).

Biochemical Testing

The colonies formed on EMB Agar were analysed by performing different biochemical tests.

Indole test: Tryptone broth was incubated with the microorganism at 37 °C for 24 hours. After incubation, Kovac's reagent was added to check the presence of the enzyme tryptophanase, which converts amino acid tryptophane to indole. Appearance of a distinct red color at the top of the layer of broth is a positive test.

Voges Proskauer Test (VP): This test is mainly used to detect organisms that produce acetyl methyl carbinol from glucose fermentation. Organisms are incubated in MR-VP broth for 48 hours. 5 drops each of Baritt A and Baritt B reagent are added to the test tubes. A positive test shows a red color after 10 to 15 minutes.

Methyl Red: This is a biochemical test that is used to detect the formation of different acids by microorganisms. Organisms are incubated in MR-VP broth for 96 hours. Appearance of a distinct red color after adding 5 drops of methyl red solution is a positive test.

Citrate: It is used to test an organism's ability to utilize sodium citrate as a source of energy. The tube of Simmons citrate agar was inoculated and incubated for 96 h at 37°C. If the medium becomes green to blue, the result is positive.

Methylene Blue Reduction Test

1ml of methylene blue was added to 10 ml of both milk samples and mixed properly. Tubes were placed in a water bath at 370 C and monitored regularly. The methylene blue reduction test is based on the bacteria in milk that grow and consume the dissolved oxygen. The speed of the colour disappearance is directly proportional to the number of bacteria present.

5. RESULT AND DISCUSSION

5.1 RESULTS

When unpasteurized and pasteurized milk was cultured on Tryptone glucose yeast agar (TGYA) off-white coloured, clear to slightly opalescent circular as well as spreader colonies (Fig.1a and 1b) were observed in unpasteurized milk sample after an incubation at 35-37°C for 24 hours whereas no growth was seen in

pasteurized milk sample. When observed under the microscope, rod shaped and cocci-shaped bacteria were observed.

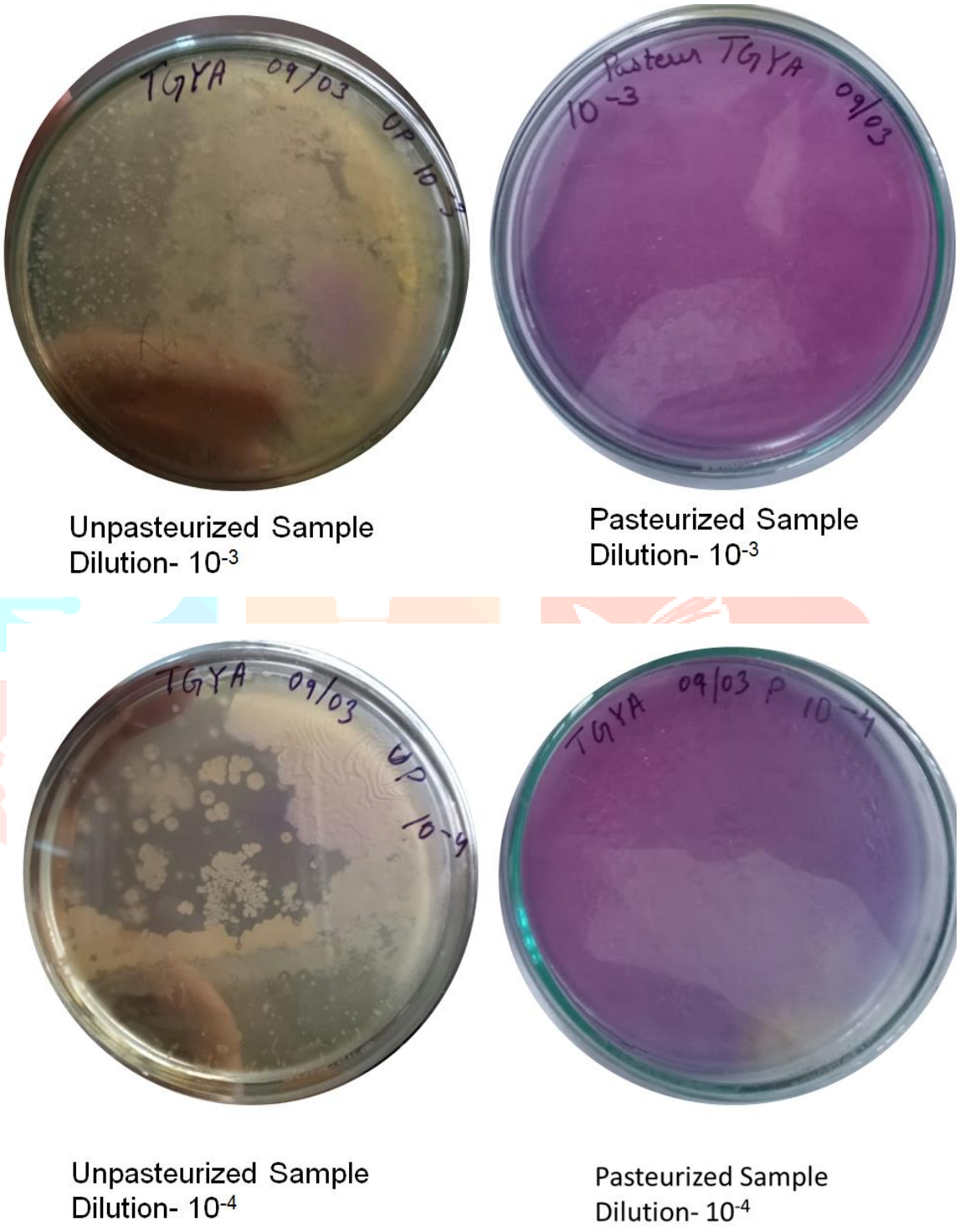


Fig.(1a and 1b). Tryptone glucose yeast agar

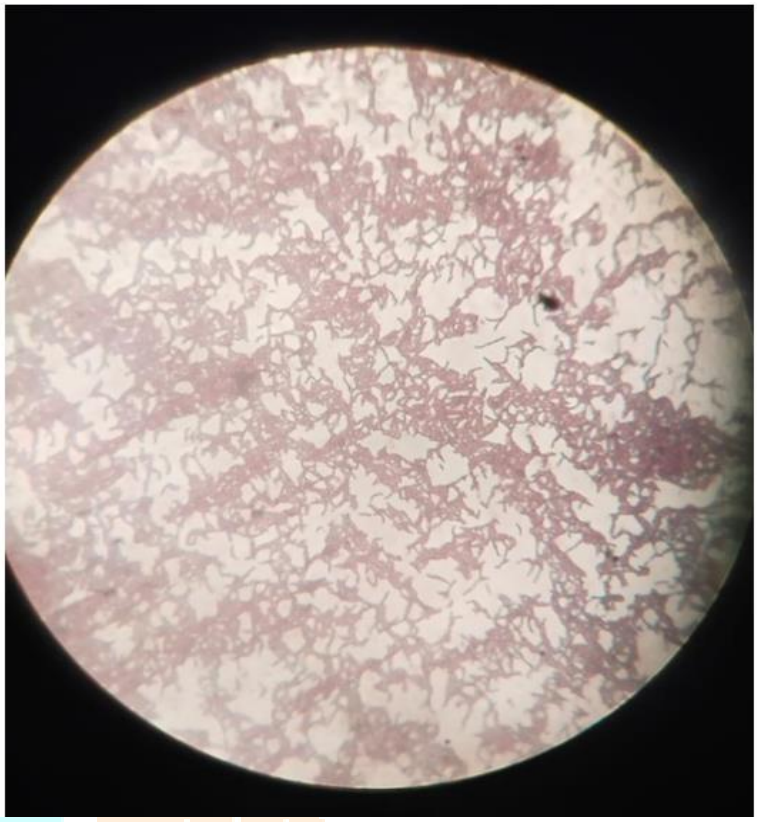


Fig.(1c). Gram staining from Tryptone Glucose Yeast Agar

Samples were cultured on MacConkey Agar medium, which is a selective and differential medium for lactose ferments and non-lactose fermenting bacteria. Pink-red colonies (Fig.2a and 2b) were observed indicating the presence of Lactose-fermenting bacteria. (Examples include *Escherichia coli*, *Klebsiella spp*, *Citrobacter, Enterobacter, etc.*). Non-lactose fermenters can't utilize lactose, therefore colonies appear colorless or transparent. The appearance of a pink halo around colonies or areas of confluent growth shows strongly lactose fermenting bacteria are present. These bacteria produce sufficient acid which causes precipitation of the bile salts around the growth. Under the microscope, rod shaped gram negative bacteria were observed after performing gram staining.(Fig.2c)

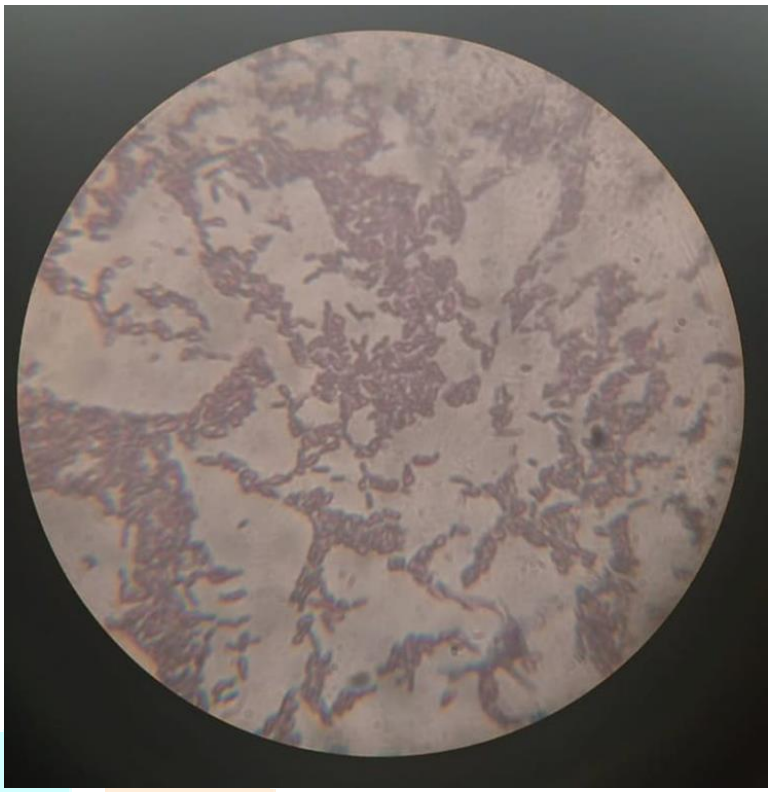
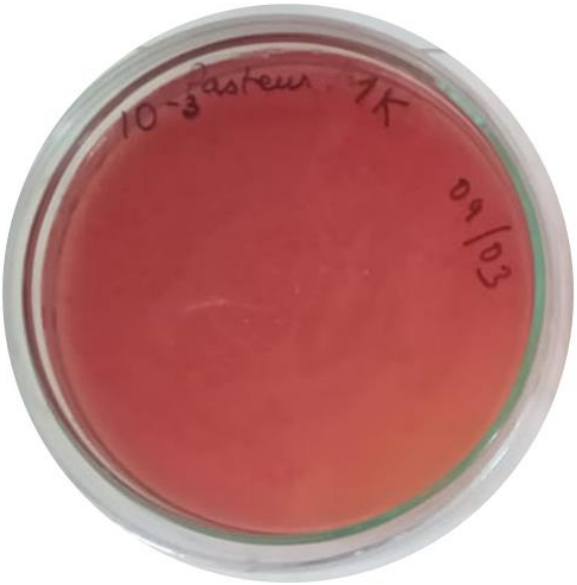


Fig.(2c) Gram staining from MacConkey Agar

Unpasteurized Sample
Dilution- 10^{-3}



Pasteurized Sample
Dilution- 10^{-3}

Fig.(2a and 2b). MacConkey Agar

Samples cultured on Eosin Methylene Blue Agar (EMB Agar) (Fig.3a). Colonies are 2-3 mm in diameter with a greenish metallic sheen in reflected light, dark or even black center in transmitted light were observed after 24 hour incubation which suggests that *Escherichia coli* might be present.

Colonies with no metallic sheen, grey-brown centers transmitted by light as well as pink-colored colonies were present in the agar. Rod-shaped gram negative bacteria were observed under the microscope.

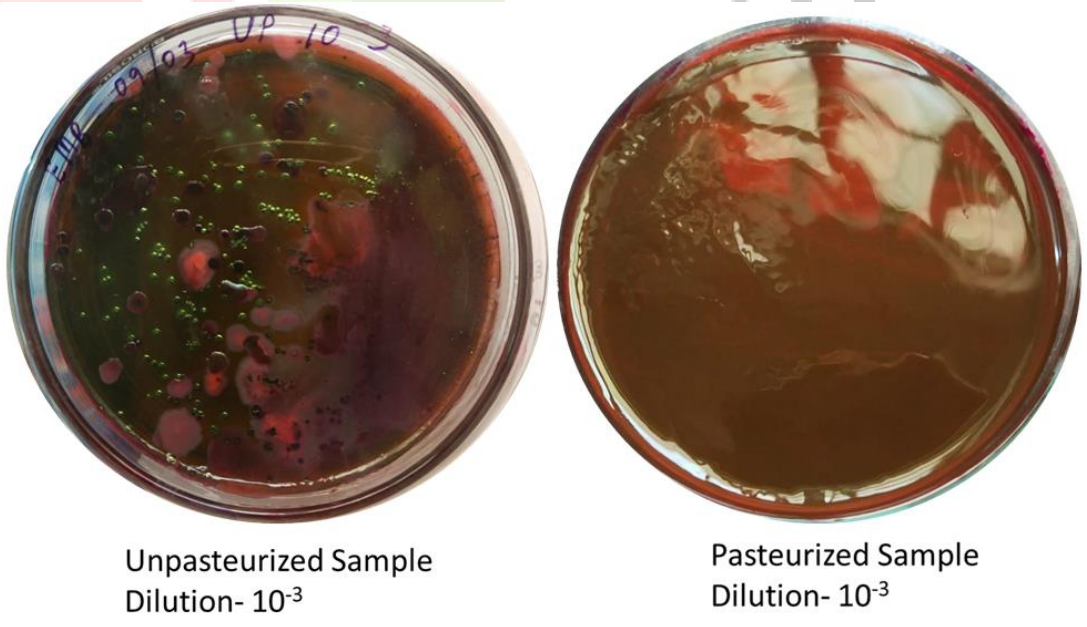


Fig.(3a) Eosin Methylene Blue Agar

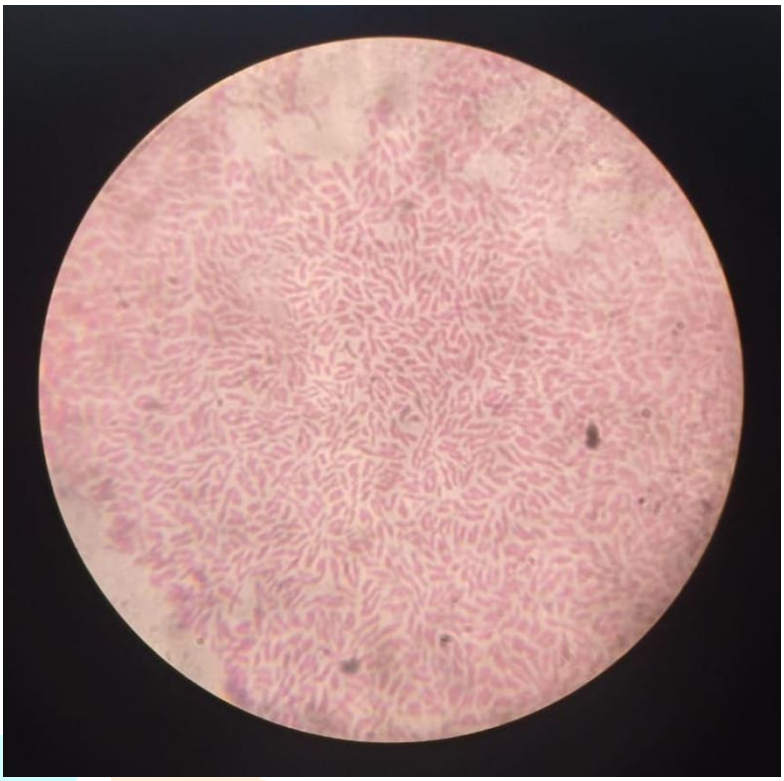


Fig.(3b) Gram staining from Eosin Methylene Blue Agar

Biochemical tests

The colonies formed on EMB Agar were analyzed by performing the following biochemical tests (IMViC).

- Indole Test
- Methyl Red Test
- Voges Proskauer Test
- Citrate Utilization Test

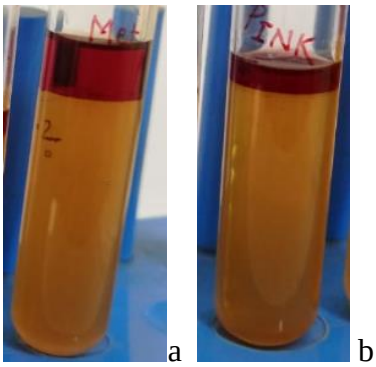
Results obtained can be seen in table 2.

Test	**Colony A**	**Colony B**
Indole Test	+	+
Methyl Red Test	-	+
Voges-Proskauer Test	+	-
Citrate Utilization Test	+	-

Table- 2 Biochemical tests results. (+) positive and (-) negative

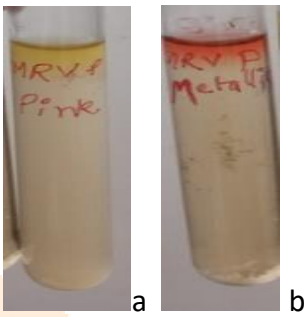
From the results, *Klebsiella oxytoca* in Colony Aand *Escherichia coli (E. coli)* in Colony B might be present.

Further biochemical tests are required to be performed.



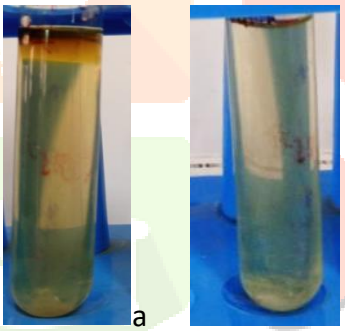
a b

Fig.(4a and 4b)- Indole Test



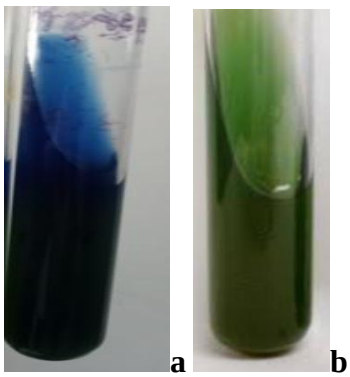
a b

Fig.(5a and 5b)- Methyl Red Test



a b

Fig.(6a and 6b)- Voges-Proskauer Test



a **b**

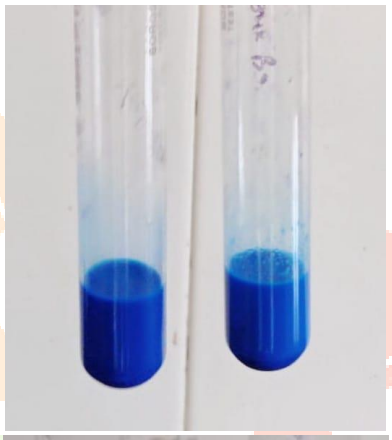
Fig.(7a and 7b)- Citrate Utilization Test

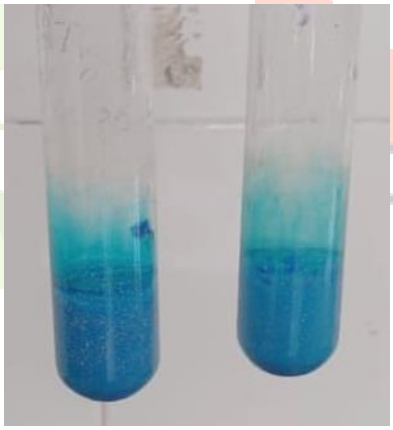
Methylene Blue Reduction Test

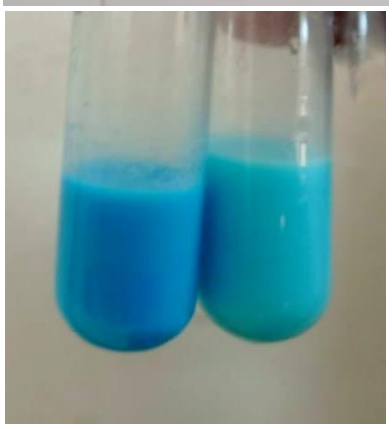
1ml of methylene blue solution was added to 10 ml of each milk sample. Tubes were placed in water bath at 37^0 C and monitored regularly. The test gave the following results in mentioned in table 3.

	Pasteurized Milk	**Raw (Unpasteurized) Milk**
Time taken for reductior	6.14 hours	5.40 hours
Result	Good quality	Fair quality

Table 3- Methylene Blue Reduction Test results

1. 

2. 

3. 

4. 

Fig.(10a, 10b, 10c and 10d)- Methylene Blue Reduction Test

5.2 DISCUSSION

Milk is a complete food and rich in all the nutrients required for the growth and development of people belonging to all age groups. Milk has essential nutrients like fat, protein, carbohydrates, minerals, vitamins. But having a high amount of nutrients puts it at the risk of spoilage, since it serves as an excellent growth medium for microorganisms.

In this study, we used an unpasteurized and a pasteurized (local brand) milk sample which we collected from local market shops in Kota City, Rajasthan. The bacterial growth in the raw milk sample indicated high contamination whereas, negligible bacterial growth was observed in the pasteurized milk sample, which suggests it is safer for consumption.

Raw (unpackaged /unheated) milk is readily available in the market and it is highly contaminated with pathogenic bacteria. There are many ways by which milk is contaminated: bacteria present in the environment, unsterile utensils, unhygienic storage tanks, dirty condition of milking hands, diseased animals etc. Animal health is also very important because a diseased cow means loss in quantity and quality of milk production. Farmers should maintain good hygiene, satisfactory nutrition, pasture rotation, and treat animals against parasites at fixed intervals.

According to different studies world-wide, unpasteurized /raw milk is not healthy for human consumption until it is not properly boiled. Pathogenic bacteria present in the environment, milk and milk products are easily transmitted into the human body, which is a matter of public health concern. Commonly found pathogens such as *Campylobacter jejuni, Coliform bacteria, Escherichia coli, Listeria monocytogenes, Mycobacterium bovis or tuberculosis, Salmonella spp.* in milk can cause diseases like Gastroenteritis,

Hemolytic uremic syndrome (HUS), Listeriosis, Typhoid fever, Tuberculosis etc. The presence of such harmful bacteria in an item for daily consumption is gravely concerning.

High water content in milk is one of the factors that allow pathogenic bacteria to grow. Water is the most common adulterant added to milkthroughout the world. This is done possibly to fill the demand and supply gap, to increase the quantity of food production & sales, due to lack of efficient food law and government initiatives, to fulfill trader's profit motivation and many other reasons.

Louis Pasteur spent a part of his career researching how bacteria could sour beverages. His work was mainly focused on beer and wine, but he also discovered the similar properties in milk. To forestall spoilage, Pasteur invented a process to eliminate bacteria by heating and cooling milk, which is now called pasteurization. Pasteurization is a process which is undertaken by dairy companies that sell pasteurized milk in packets.The strategy involves heating milk at a selected temperature for a stipulated time to kill harmful bacteria which will cause severe damage to human health. The U.S FDA and the Centers for Disease Control and Prevention (CDC) state that "pasteurization does not reduce milk's nutritional value". Ultimately, pasteurization of milk makes it safer for consumption, because the tactic works to kill potentially dangerous bacteria that are accountable for causing numerous food-borne diseases.

Different tests performed in this study inform us that pasteurized milk is very safe to consume and how important it is to maintain hygienic conditions while milking animals, storing and transporting milk.

6. CONCLUSION

We observed very high bacterial growth in three different agars containing unpasteurized milk sample while almost no bacterial growth in agars containing pasteurized milk. The biochemical tests on two colonies of EMB agar indicated the presence of two species of microorganisms - *Escherichia coli (E. coli) and Klebsiella oxytoca.* In this study, methylene blue reductase test was also performed for the detection of the quality of milk sample. Pasteurized milk took about 6.14 hours for reduction which indicates it is good in quality whereas unpasteurized milk sample took 5.40 hours for reduction which suggests it has lower quality than pasteurized milk.

From the results we conclude that unpasteurized milk has much more contamination than pasteurized milk. Therefore strict hygienic measures should be taken during production, processing and distribution of milk to avoid contamination. Time to time inspection must be done by specialists on the dairy farms to minimize milk contamination with different types of microorganisms. Efficient cleaning and sanitization of dairy utensils must be done to improve the quality of raw milk. The milk should be kept under refrigeration at all times and the practices of adulteration of milk for trader's profits should be discouraged.

Also initiatives should be taken towards informing people about the harmful effects of raw milk consumption and importance of pasteurization. Proper time and temperature should be maintained while pasteurizing milk to eliminate disease causing organisms and any other contamination.

7. SCOPE FOR FURTHER WORK

Some limitations in this study could have been the limited number of samples, and that the samples were taken only from a specific location in the Kota city. Therefore, in future a bigger sample size can be taken from different locations in the city or from different states for comparison. Level of adulteration should also be checked for the obtained samples. A comparative study between different brands of pasteurized milk can also be done which will help consumers choose the brands with the best quality milk. Some more biochemical tests can be performed in order track down the presence of specific pathogenic bacterial species.

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