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# RESEARCH ARTICLE

## MICROBIAL QUALITY AND CHEMICAL ADULTERANTS EVALUATION IN THE RAW AND PASTEURIZED MILK

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**ABSTRACT**

Milk is considered as a complete diet as it contains the major elements required for the growth and maintenance of body like proteins, fat, sugar, minerals and vitamins but it has been observed that it also acts as suitable medium/vehicle for the pathogenic/spoilage microorganism. It is also likely to be contaminated intentionally/unintentionally at various level of production, processing and marketing. Therefore, the present study was conducted with an objective to assess the quality of market milk available in and around Ludhiana. Fresh and pasteurized milk sample were collected hygienically and subjected to various physico-chemical and microbiological analysis. Among all the adulterant tested in raw milk 10% sample are positive for starch and 20% positive for carbonate, however pasteurized milk sample negative for all test. Results for the methylene blue reduction test (MBRT)30% poor, 10% excellent, 50% fair and 10% are good for raw milk samples, however for pasteurized sample 40% are excellent and 60%are good. For the isolation of the microorganism in both raw and pasteurized sample *Salmonella* 9.1%, *Proteus* 13.6%, *E. coli* 31.8%, *Klebseilla*18.2% unidentified 13.6% and no coliform 13.6% are positive.



## INTRODUCTION

Milk has an outstanding nutritional quality. It is an important food of diet of vast population on earth. People of all age groups usually use milk and milk products as a dietary and high-grade food. Milk is considered as a complete diet as it contains the major elements required for the growth and maintenance of body like proteins, fat, sugar, minerals and vitamins etc. It is exceptionally important for the growing children. Approximately 50% of the milk produced is consumed as fresh or boiled, one sixth as yoghurt or curd and remaining is utilized for manufacturing indigenous variety of milk products such as ice cream, butter, khoa, paneer, rabri, kheer etc. (Anjum *et al.,* 1989). The simplicity and rapidity with which milk can be adulterated has always tempted unscrupulous milk vendors to indulge in fraudulent practices and adulteration of the milk (Ali Ahmed., 2009). The ever increasing greed has given way to a new type of adulterated milk called synthetic milk which exactly looks like the natural milk, has same specific gravity, fat and Solid not fat (SNF) value and is prepared by mixing water, pulverized detergent or soap, sodium hydroxide, vegetable oil, salt and urea. It is very dangerous from health point of view. The use of synthetic milk has been found to have cancerous effects on human beings.

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Urea and caustic soda are very harmful to heart, liver and kidneys. Urea is an additional burden for kidneys as they have to do more work to remove urea from the body. Caustic soda which contains sodium acts as slow poison for those suffering from hypertension and heart ailments. Caustic soda also deprives the body from utilizing lysine, an essential amino acid in milk, which is required by growing babies. Such artificial milk is harmful for all, but is more dangerous for pregnant women, fetus and persons, who are already having heart and kidney problems. At the same time milk is the medium that ensures growth of microorganisms when suitable temperature exists. If it is produced un-hygienically and handled carelessly, it gets contaminated very easily leading to its early spoilage (Oliver *et al.,* 2005). The quality of milk is determined by aspect 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 multiplication of many kinds of micro-organisms. Milk is an efficient vehicle for transmission of diseases to humans. Approximately 90% of dairy related diseases in human being arise from unhygienic milk products (Ryser, 1998). To protect public health against milk-borne infections, there are regulations that require proper hygienic handling of milk and its pasteurization. Bacteriological contamination of raw milk can occur either from contamination of milk from environmental sources especially contamination during the milking process or from shedding of mastitis organisms from within the udder (Reinemann *et al*., 1997). Recent studies have

established the emergence of new milk-borne bacterial pathogens such as *Escherichia coli* O157:H7 with more serious challenges for public health and the diary industry. Raw milk may be contaminated by a wide range of bacteria, including *Staphylococcus aureus (Romilda Castro Cairo et al., 2008), E. coli (Parekh and Subhash, 2008), Pseudomonas pyocyaneus (Kumaresan and Villi, 2008), Corynebacterium* spp and *Bacillus*. Raw milk has been repeatedly demonstrated as the vehicles for a variety of human pathogens, including *Campylobacter*, *Streptococcus zooepidemicus*, and *Salmonella* of many different serotypes. The present study will be envisaged to assess the microbial and chemical quality of raw and pasteurized milk.

## MATERIALS AND METHODS

The samples were collected from various vendors, pasteurized milk samples of five different brands were collected from different sources in the sterile bottles, sealed properly, brought to the laboratory, kept at below 4°C and tested within three to four hours after collection. All the samples were evaluated for the detection of adulterants, microbial quality by standard plate count (SPC) then presumptive test were performed to observe the presence of lactose and non-lactose fermenting bacteria by using MacConkey agar. The samples were inoculated on MacConkey Agar and incubated aerobically at 37$^{o}$C for 24 hours. Then non-lactose fermenting colonies were sub cultured again on MacConkey agar for purification of the isolate. Gram staining was performed to ensure the purity of the organism. Salmonella was identified by various biochemical tests e.g. Catalase test, Simmon Citrate Agar, Indole Production, Nitrate reduction, Urease production, Voges Proskaur, Methyl red. Simultaneously all the milk samples (raw and pasteurized) were evaluated for their biochemical characteristics like phosphates activity, hydrolytic activity, presence of adulterants like carbonates and bicarbonates, boric acid and borates, formaldehyde, urea, detergent and carbohydrates etc. by using standard methods.

### Methylene Blue Reduction Test (MBRT)

1 ml of methylene blue was mixed with 10 ml of milk. The tube was sealed with rubber stopper and slowly inverted three times to mix the sample thoroughly. It was placed in water bath at 35$^{o}$C and examined at intervals upto 8 hours.

### Standard Plate Count

1 ml of dilutions (milk/milk product) was added to Petri plates and nutrient agar medium was poured in it. Triplicate plates were prepared and incubated at 37°C for 48 hours. Plates showing colonies between 30-300 were selected for total count. The average count of two plates was taken for the standard plate count/ml/gram

### Presumptive coliform test

1 ml of milk samples (raw and pasteurized) were mixed with 9 ml of sterile distilled water and serial dilution were made upto $10^{-5}$ to $10^{-6}$. 1 ml of dilutions were added to petri plates with McConkey agar medium in triplicates and incubated at $37^{0}$ C for 48 hours.1 ml of each dilution was transferred to McConkey broth for the detection of acid production and formation of gas.

### Biochemical Characteristics

### Production of Catalase

This illustrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. Few drops of three percent hydrogen peroxide were added to 1 drop of bacterial culture. Formation of bubbles is a positive test for catalase.

### Production of Oxidase

This test depends on presence of certain oxidases in bacteria that will catalyse the transport of electron donors in bacteria. Few drops of oxidase reagent were added on the bacterial culture grown on nutrient agar medium. Change of colony colour to pink and finally to black is the positive indicator of the presence of oxidase.

### Nitrate Reduction Test

This test is used to differentiate the members of family enterobacteriaceae from other gram negative bacteria that do not produce the nitrate reductase enzyme. This enzyme reduces nitrates into nitrite. The test was done by inoculating heavy growth of test organism into 2.0 ml nitrate broth. After 4-6 hour incubation at $37^{0}$C, one drop of sulphanilic acid and one drop of alpha- naphthylamine was added. Development of red color after mixing was taken as positive.

### IMVIC test

### Test for Indole Production

This test demonstrates the ability of certain bacteria to decompose amino acid tryptophan to indole which accumulates in the medium. The test was performed by inoculating the culture into tryptone broth and incubating for 24±2 h at 35°C. 0.2-0.3 mL of Kovac's reagent was added into the tubes. Appearance of distinct red color in upper layer is the positive test.

### Voges-Proskauer (VP)-Reactive Compounds

This test indicates the production of acetyl-methyl carbinol in peptone water containing glucose by some bacteria. The test was performed by adding few drops of VP solution (3 ml 5% alpha –naphthol + 1 ml 40% KOH) into 24- 48 hr cultures grown in MR-VP medium. Test is positive if pink color develops while no color change was observed in negative cases.

### Methyl Red-Reactive Compounds

After VP test, MR-VP tubes were incubated for additional 48±2 h at 35°C. After that, 5 drops of methyl red solution was added to each tube. Distinct red color indicates positive whereas Yellow indicates negative reaction.

### Citrate Utilization Test

This test demonstrates the capacity of organisms to use citrate as sole source of carbon and ammonium salt as sole source of nitrogen. Slants of Simmon's citrate agar medium were prepared and bacterial culture was streaked on it. Slants were incubated at $37^0$C for 48 hours. Development of blue color due to utilization of citrate is a positive test.

## RESULTS AND DISCUSSION

The present investigation was taken up to study the quality of raw and pasteurized milk chemically as well as microbiologically. In the present study milk samples were examined for the presence of adulterants. About 10% (one sample) of raw milk sample were found to be positive for starch whereas20%samples (two) exhibited the presence of carbonates. None of the pasteurized milk samples showed the presence of any adulterants. Absence of any adulterant in the pasteurized milk indicates the strict laboratory testing standards followed by the companies to build their brand image (Table 1). Methylene blue reduction test (MBRT) performed for raw milk revealed that out of ten samples three were poor, five were fair, two were good and one was excellent and out of five samples of pasteurized milk three were good and two were excellent (Table 2). MBRT is a fast method to check microbial quality of the samples. But the results obtained with MBRT are not in cognizance with those obtained with standard plate count.

It is not a very reliable method for detection of contamination in dairy products (Igumbor *et al* (2002). This method should be used in combination with other methods to assess bacterial quality. The total viable count of raw milk ranged from 2.4 x $10^4$ to 1.36 x $10^7$ cfu/mL (Table 3). This figure can be regarded as a high count as mentioned by Bramley and Mckinnon (1990) that counts of greater than$10^5$cfu/ml for raw milk are indicative of serious faults in hygiene production of milk. There is an urgent need to educate the farmers and milk vendors about the clean milk production practices. Milk may get contaminated with different micro-organisms due to direct or indirect contact with any source of external contaminants during the steps of milking, collection and transport. Direct physical contact of milk with unclean surfaces such as milking utensils, udders and teats, and hands of handlers besides environmental factors such as design and cleanliness of building and installations, the adequacy of water supply, the manner in which dung and other wastes are disposed off and the amount of dust in the immediate surroundings are important in so far they may contribute to microbial contamination of surfaces with which milk comes in to contact. Pasteurized milk of different brands showed different values of standard plate count. It ranged from 8.3 x $10^3$ to 1.5 x $10^5$cfu/ml. Post-pasteurization contamination may occur due to improper handling of milk or leakage of packaging or fractured cold chain. According to Pasteurized Milk Ordinance(PMO) standards, SPC of Grade A milk for an individual producer should not exceed 100,000 cfu/mL (http://www.google.com/patents/ P2391222A2?cl=en).

**Table 1. Chemical adulterants in raw and pasteurized milk**

| S No | Starch | Sugar | Boric acid /borates | Carbonates | Formaldehyde | Urea | Detergent | Phosphatase activity |
|---|---|---|---|---|---|---|---|---|
| | | | | | Raw Milk | | | |
| 1 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 2 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 3 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 4 | -ve | -ve | -ve | +ve | -ve | -ve | -ve | +ve |
| 5 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 6 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 7 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 8 | +ve | -ve | -ve | +ve | -ve | -ve | -ve | +ve |
| 9 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| 10 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| | | | | | Pasteurized Milk | | | |
| 1 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| 2 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| 3 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| 4 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| 5 | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |

**Table 2. Decolorizing time and grading of milk samples by MBRT test**

| SAMPLE TYPE | SAMPLE NUMBER | DECOLORIZATION TIME | GRADE |
|---|---|---|---|
| Raw Milk | 1 | 5.15h | Fair |
| | 2 | 2.3h | Fair |
| | 3 | 6.4h | Good |
| | 4 | 1.25h | Poor |
| | 5 | 1.45h | Poor |
| | 6 | 4.35h | Fair |
| | 7 | 2.28h | Fair |
| | 8 | 5.25h | Fair |
| | 9 | >8h | Excellent |
| | 10 | 1.35h | Poor |
| Pasteurized Milk | | | |
| | 1 | >8h | Excellent |
| | 2 | 6.25h | Good |
| | 3 | 7.15h | Good |
| | 4 | 7.30h | Good |
| | 5 | >8h | Excellent |

**Table 3. Enumeration of micro-organisms in milk samples by standard plate count method (SPC)**

| SAMPLE TYPE | SAMPLE NUMBER | Cfu/ml($10^4$) |
|---|---|---|
| Raw Milk | 1 | $8.3 \times 10^6$ |
| | 2 | $1X10^6$ |
| | 3 | $5 \times 10^4$ |
| | 4 | $2.1 \times 10^5$ |
| | 5 | $1.36 \times 10^7$ |
| | 6 | $1.6 \times 10^6$ |
| | 7 | $1.2 \times 10^6$ |
| | 8 | $7.9 \times 10^4$ |
| | 9 | $2.4 \times 10^4$ |
| | 10 | $1.93 \times 10^5$ |
| Pasteurized Milk | 1 | $1.5 \times 10^5$ |
| | 2 | $8.3 \times 10^3$ |
| | 3 | $1.9 \times 10^4$ |
| | 4 | $2.7 \times 10^4$ |
| | 5 | $2.3 \times 10^4$ |

**Table 4. Results of presumptive test (Raw and Pasteurized Milk)**

| S No. | Cfu /ml (1/1000) | Lactose fermenting | Percentage | Non-lactose fermenting | Percentage |
|---|---|---|---|---|---|
| | | | RAW MILK | | |
| 1 | 28 | 28 | 100 | 0 | 0 |
| 2 | 24 | 17 | 70.8 | 7 | 29.2 |
| 3 | 32 | 14 | 43.8 | 18 | 56.2 |
| 4 | 45 | 23 | 51.1 | 22 | 48.9 |
| 5 | 37 | 26 | 70.2 | 11 | 29.8 |
| 6 | 10 | 10 | 100 | 0 | 0 |
| 7 | 17 | 17 | 100 | 0 | 0 |
| 8 | 51 | 23 | 45.1 | 28 | 54.9 |
| 9 | 0 | 0 | 0 | 0 | 0 |
| 10 | 28 | 28 | 100 | 10 | |
| | | | PASTEURIZED MILK | | |
| 1 | 15 | 15 | 100 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 |
| 3 | 12 | 8 | 66.7 | 4 | 33.3 |
| 4 | 18 | 18 | 100 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 |

**Table 5. Number of Lactose and Non-Lactose Fermenting Bacteria (Presumptive test)**

| Particular of sample | No. of sample | Total No. of Coliforms (per ml per g) | | | Lactose Fermenting | | | Non-lactose Fermenting | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Avg. | Max | Min | Avg. | Max | Min | Avg. | Max | Min |
| Raw Milk | 10 | 27200 | 51000 | 10000 | 18600 | 28000 | 10000 | 9600 | 28000 | 7000 |
| Pasteurized Milk | 5 | 900 | 1800 | 1500 | 820 | 1800 | 800 | 80 | 400 | 0 |

**Table 6. Result of isolation of micro-organisms from various milk samples**

| S.NO. | TYPE OF MICRO-ORGANISM | RAW MILK | PASTEURIZED MILK | TOTAL |
|---|---|---|---|---|
| 1 | Salmonella | 2(12.5%) | 0 | 2(9.1%) |
| 2 | Proteus | 2(12.5%) | 1(16.7%) | 3(13.6%) |
| 3 | E.coli | 5(31.3%) | 2(33.3%) | 7(31.8%) |
| 4 | Klebsiella | 4(25%) | 0 | 4(18.2%) |
| 5 | Unidentified | 2(12.5%) | 1(16.7%) | 3(13.6%) |
| 6 | No Coliform | 1(6.2%) | 2(33.3%) | 3(13.6%) |
| | Total no. of samples | 16 | 6 | 22 |

In the present study one sample out of five (20%) showed more than $10^5$cfu/mL. Elevated numbers of bacteria in milk generally arise from at least one of 4 common sources: dirty teats, soiled equipment, mastitis infections, and poor refrigeration (Murphy and Boor, 2000). From the results of present study it was found that most of the samples were contaminated with coliform bacteria. Table 4 showed the average number of coliforms, the minimum and maximum number of coliforms in each sample. These were then categorized as lactose ferment or and non-lactose ferment or. Table also shows the average, the maximum and the minimum number each of lactose fermentor and non-lactose fermentor. Coliforms were found both in raw and pasteurized milk but raw milk had higher contamination (90%) than pasteurized milk (60%). Raw milk showed an average coliform count of 27,200/mL whereas pasteurized milk showed count of 900/mL (Table 5). According to Harrigan and McCance (1976), coliform bacteria count should be less than 200 cfu/g in food. The existence of the coliforms has been considered as leading to the fact that the product was subject to process under inefficient hygienic conditions (Harrigan and McCance, 1976; Altug and Bayrak, 2003). In the present study out of 35 samples of milk and milk products examined, 28 samples showed the presence of coliform. 45 isolates were obtained from these 28 samples which were further characterized as *E.coli*, *Klebsiella*, *Proteus* and *Salmonella* on the basis of morphological and biochemical identification. Among them *E.coli* were predominant (26.9%), *Klebsiella* (25%), *Proteus*

(11.5), *Salmonella* (9.6%) and 13.5% were unidentified. About 93.8% of raw milk samples in the present study were found to contain coliforms, which were further categorized as *Salmonella* (12.5%), *Proteus* (12.5%), *E.coli* (31.3%) *Klebsiella* (25%) and 12.5% were unidentified. In case of pasteurized milk coliforms were present in 66.7% of samples. None of the sample contained *Salmonella* and *Klebsiella,* whereas 16.7% and 33.3% samples had *Proteus* and *E.coli* respectively. Total 16.7% samples had unidentified coliforms. Parekh and Subhash, (2008) had isolated *E.coli* from raw milk as high as 45%; Bashir and Usman (2008) as 27% and Soomro *et al*., (2002) as high as 57%. In other cases E. *coli* O157:H7 was found in 0.87 to 10% of the bulk tank milk samples tested (Ekici *et al.*, 2004; Oksuz *et al*., 2003). 33.3% of pasteurized milk samples were detected positive for *E.coli*. Da Silva *et al*., 2001 isolated 208 strains of *E.coli* from 90 pasteurized milk samples, out of which 46(22.1%) were entero pathogenic. In 2003, Ferral Berndt isolated *E. coli* from 17% of pasteurized milk samples. Its occurrence in milk may be due to improper milking, handling and inferior quality of water (Fook *et al*., 2004).

Salmonella has been detected in 12.5% of raw milk samples in the present study. The prevalence of *Salmonella* spp. has been reported for bulk tank milk samples in individual states for California, Michigan, Minnesota, Missouri, New Mexico, New York, Ohio, Pennsylvania, South Dakota, Tennessee, Texas, Washington etc. *Salmonella* spp. were found in 0.17 to 8.9% of the bulk tank milk samples tested (Desmasures *et al.*, 1997; Rohrbach *et al.*, 1992), indicating the widespread presence of *Salmonella* in unpasteurized milk. Bashir and Usman (2008) isolated Salmonella from 17% of the raw milk samples analyzed. However Murry (1966) could not isolate *Salmonella* from bulk collected milk samples in Northern Ireland but *S.dublin* was isolated from two individual producers. None of the pasteurized milk sample examined was found to contain Salmonella. These findings are in accordance with Khalilur Rahman and Abdul Malik (2002). The absence of *Salmonella* isolation from milk samples in spite of unhygienic practices employed in milk production could be due to the fact that in some cases salmonellae in milk might escape detection because of their usually small number or possibly, the growth of *Salmonella* might have been inhibited by other fast growing organisms. The isolation of salmonellae by other workers might be due to the adulteration practices which are very commonly used by milk vendors by using contaminated water. Raw milk was detected with 25%*Klebsiella* and 12.5% *Proteu*s. Donkar *et al*., 2002 isolated *Klebsiella* from 16.7% samples and *Proteus* from 7.3% raw milk samples. *Klebsiella* was not detected in pasteurized milk in the present study but Igumbor *et al*. (2002) isolated *Klebsiella* from pasteurized milk samples.

**Conclusions**

In present study by the above observation and finding, it can be concluded that the gap between demand and supply is one of the important reason for the milk adulteration. Unhygienic milking, handling of milk, failure in cold chain maintenance during transport, post pasteurization contamination, personal and utensil hygiene and contaminated adulterated ingredients are the main factors responsible for the microbial deterioration of the milk.

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