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Microbiological analysing milk samples obtained from Indian manufactures

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ABSTRACT

Milk is one of the most consumed food items by the Indian population. When first obtained from the udder of a healthy cow, it is usually sterile. However, due to its high nutrient content and thus role as a highly nutritious medium for growth, it quickly gets contaminated by microbes, especially bacteria. Some of these microbes may also give rise to bowel diseases. The microbiological quality of milk may deteriorate with time due to several factors like unhygienic milking methods, poor hygiene of handlers and improper conditions of storage. Coliforms have always been used to determine the quality of raw and pasteurized milk. In this study, we try to analyze the microbial quality of the milk samples obtained from different Indian manufacturers, by performing four tests, namely MBRT, Phosphatase Test, Coliform Test and SPC.

Coliforms were detected in a few packaged milk samples.

KEYWORDS: Milk, Delhi, Coliforms, Microbiology, MBRT, Phosphatase, SPC.

1. INTRODUCTION

Milk and milk products are important components of daily diet intake of consumers all over the world [1]. They consist of nutrients that are essential for both a healthy body and a healthy mind. Dairy industry requires both skilled and unskilled labour for its operations like milking and marketing of milk, maintenance of animals, feeding etc. The dairy industry is, mainly, being handled by three groups. The first being the farmers, who produce the milk, then comes the processors, who convert raw milk into whole and lower fat milk and the third category is the retailers, who sell the final products [2].

The main aim of the Indian dairy industry is to better manage the national resources in order to enhance milk production and upgrade milk processing by using innovative technologies. It is

considered as one of the activities aimed at alleviating the poverty and unemployment especially in rural areas and in the rain fed and drought prone areas [3]. The growth and expansion of dairy industry has opened up job opportunities in recent years especially in developing countries [4]. Dairy industry contributes about 5.3% to India's agriculture GDP. This sector engaged 120 million rural families in milk production activities. After getting a great boost from the production sector, now the quality and hygienic conditions for production is to be taken care of for the consumers [5].

Milk is a nutrient rich liquid food derived from healthy mammals. It should be free from colostrum. Total urea in the milk shall not be more than 700 ppm [6]. Seventy five percent of milk produced in animal husbandry is used in the liquid form. A large quantity of this is pasteurized. Pasteurization is a heat treatment process designed to reduce or destroy the number of pathogens of raw milk to such an extent that it is does not constitute any health problem. Pasteurization is a two way process consisting of rapid heating of milk followed by immediate cooling [7]. This process helps in increasing the shelf life of milk and reducing the number of microorganisms whereas, the direct consumption of raw milk eliminates the nutrient losses that Unprocessed raw milk can be stored at room temperature without spoilage for up to 8-hours [9]. The pasteurization of milk can increase the shelf life of milk and also the consumer's needs would be fulfilled through hygienic and high quality pasteurized milk [10].

Microbial quality of milk

Milk being a highly nutritious and valuable human food is consumed daily by millions of people in variety of forms/products. The nutrient composition of milk is not constant and shows a wide variation [11]. This property of milk, however, makes it an ideal medium for bacterial growth and making it one of the most perishable agricultural food products because it can be contaminated very easily, as shown in Table 1.1. The milk composition can be affected by genetics, stage of lactation, udder health, level of milk production, season, age of milk animal and environmental temperature [12].

The lactose in milk is used by group of lactic acid bacteria and convert lactose into lactic acid. It belongs to the family of *Streptococcus lactis*. They grow very fast and multiply when milk is kept in ambient temperature. It causes the natural souring of milk. The source of these

bacteria is air and dust etc. which is present in the environment. How soon the milk turns sour depends on the degree of contamination and on the temperature of milk **[13]**.

The total bacterial count in milk decides the quality and safety of dairy products **[14]**. Contamination of milk by bacteria is not suitable for further processing as it falls short of the expectations of consumers in terms of health (nutritional value), satisfaction (sensory attributes) andare due to heat treatment in milk. The commercially distributed raw milk is harmful due to safety reasons **[8]**. safety (hygienic quality) **[15]**. Many contaminating organisms only spoil the products, thereby, reducing its shelf-life, while others, are pathogenic to man and can transmit diseases if the milk is consumed untreated. As a result, total viable bacterial count has become one of the accepted criteria for grading milk intended for consumption or for processed dairy products **[16]**.

The importance of contaminating agents in milk-borne diseases has increased. However, more than 90% of all reported cases of dairy related illnesses continued to be of bacterial origin. Major pathogens that are frequently involved in the food borne outbreaks associated with the consumption of milk include *Listeria monocytogenes, Salmonella, Campylobacter, Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Clostridium botulinum*. The presence of these pathogenic bacteria in milk emerged as a major public health concerns, especially for individuals who still drink raw milk **[13]**.

Quantification of viable cells is a critical step in almost all the quality checks in biological experiments. A Methylene Blue dye Reduction Test (MBRT) is also used to quantify viable cells in milk based on reduction of methylene blue dye in cell cultures and is correlated well with colony forming units (CFU). The unity of the developed assay to monitor CFU rapidly and accurately for *Escherichia coli, Bacillus subtilis* and a mixed culture of *E. coli* and *B. subtilis* has been demonstrated **[17]**. Other researchers suggested that beside water quality and udder infection, hygiene with respect to containers cleaning, hand washing and disinfection were the key areas of relevance to milk hygiene intervention **[18]**.

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Milk Sample	Spoilage microorganism or microbial activity
Raw milk	Many different microbes
Pasteurized milk	Psychrotrophs, sporeformers, microbial enzymatic degradation
Concentrated milk	Spore-forming bacteria, osmophilic fungi
Dried milk	Microbial enzymatic degradation

Table 1.1: Typical types of spoilage microorganisms or microbial activity [12]

Numerous studies showed that consumption of contaminated raw milk can lead to serious human diseases like tuberculosis and brucellosis [19]. In both the diseases, the causative organisms *Mycobacterium tuberculosis* and *Brucella abortus* respectively, may be excreted in milk from infected animals. *Staphylococcus aureus* had been found to dominate the mastitis associated organisms. This poses direct threat to public health, because a proportion of bovine strains of *S. aureus* produce enterotoxins in food, the consumption of which leads to food poisoning [20].

Other investigators analyzed the samples of raw milk from cow, goat and sheep regarding pathogenic microorganisms like *Staphylococcus aureus* and *Escherichia coli* to show that the microorganisms found in milk can directly affect the human health and can cause a public illness if the unpasteurized milk is used in the food industry [21].

2. MATERIALS AND METHODS

1. MBRT Test

MBRT test was conducted using methylene blue dye. 10 ml of milk sample was taken in a sterilized test tube under aseptic conditions. 1ml methylene blue solution was added and mixed well. Capping was done with sterilized rubber cork. Test tube was transferred to water bath at 37^{O}C. It was observed after every 15 minutes up to 6 hours for the disappearance of colour. More time taken for discoloration indicated good quality.

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2. Phosphatase Test

5 ml phosphatase dye was taken in a sterilized test tube. 1 ml of milk sample was added to the dye. Capping was done with a sterilized rubber cork. Test tube was transferred to the water bath at 37^{o}C. After every 20 minutes the colour was observed. Appearance of yellow color indicated presence of enzyme (positive). If otherwise, i.e.
no appearance of yellow color indicated the absence of enzyme.

3. **Coliform Test**

Coliform count was taken. 9 mL sterile Normal Saline was taken aseptically in six sterile test tubes and these tubes were marked as 1:10, 1:100, and 1:1000 (10^{-1}, 10^{-2} and 10^{-3}) and so on to make serial dilutions of the milk sample. A sterile pipette was taken and 1 ml of the sample was aseptically transferred after thorough mixing to the test tube labelled 1:10. The pipette was discarded after use.

Another sterile pipette was taken and the contents of 1:10 test tube were mixed and then 1 ml from this tube was transferred to the next tube labelled 1:100. Further tenfold serial dilutions were made in the same manner up to 10^{-6} using fresh sterile pipette each time, without touching the NSS or wall of the test tube with the tip of the pipette. Number of dilutions was reduced in case of heat processed milk samples which were expected to carry less bacterial load.

Similarly, the number of serial dilutions was increased in case of samples expected to carry higher bacterial load. Two sterile Petri dishes were taken for each dilution and the sample number, dilution, date etc. were marked on the base of the petri plate.

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A fresh sterile pipette was taken and after thorough mixing 1ml of 10^{-6}dilution (or the highest dilution) of the sample was poured in each of the two Petri dishes corresponding to that dilution of the dish. One mL of the lower dilutions (10^{-5}, 10^{-4}, 10^{-3} and so on) of the sample was transferred to the corresponding Petri dishes in a similar manner. The same pipette can be used if transferring is started with the highest dilutions and moving to the lower dilution serially.

15-20 ml (approximately) of VRBA culture medium (melted and held at 45 in water bath) was poured to each petri-dish and the sample and the culture medium was mixed by giving gentle rotation to the petri dish on a horizontal plane. The plates were kept at room temperature and allowed to solidify.

The Petri dishes were incubated in the inverted position (bottom side up) in an incubator at 37 for 24 hours. The number of colonies appeared after incubation was counted in each plate irrespective of shape and size of the colonies.

The CFU/mL was calculated by multiplying the colonies appeared on the plate with the corresponding dilution factor to obtain the bacterial count per mL in the sample.

$$\text{CFU/ mL} = \frac{number\ \ of\ colonies\ \ x\ diluted\ \ factor}{Volume\ \ sampled}$$

4. SPC Test (Standard Plate Count)

SPC was taken using PCA (Plate Count Agar) media. The methodology was same as used for coliform test (2.1.3). The only difference was that plate count agar medium was used for SPC test. After incubation, the number of colonies was counted in each plate irrespective of shape and size of the colonies. The number of colonies was multiplied with the corresponding dilution factor to obtain the SPC per ml in the sample.

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3. RESULTS AND DISCUSSIONS

3.1. MBRT (Methylene Blue Reduction Test)

Microbiological contamination of milk is a very serious issue, as many of these microbes are pathogens and are responsible for many food-borne diseases. Methylene blue reduction test (MBRT) is an indicative test to check the bacterial load in milk. As per FSSAI, 2011 standard, reduction time of methylene blue in MBRT should not be less than five hours (300 minutes). Again, it is important to note that this standard is for plant level and not for milk purchased from retailers.

As it is clear from the Table 3.1, 17 (56.6%) of 30 packaged milk samples (60% for full cream milk and 53% for toned milk) were found to be below the prescribed standard i.e. reduce (decolorizes) the MB dye in less than 5 hours. The results for fresh loose milk samples were cent percent genuine i.e. take more than 200 minutes to decolorize MBRT whereas, the loose buffalo milk (93.3%) as well as loose cow milk (80%) samples were below the prescribed standards of at least 200 minutes (Figure 3.1). Other reporters also reported similar results indicating high bacterial load in milk samples, thus improper handling and storage of milk samples [22-25].

Type of Milk	Milk MBRT (Standard) Minimum	<200 min	≥ 200-300 min	≥ 300 min	Non-Confirming	Genuine	Total
Full cream	300 minutes	0	9	6	9 (60%)	6 (40%)	15 (100%)
Toned	300 minutes	0	8	7	8 (53.3%)	7 (46.6%)	15 (100%)
Loose cow milk	200 minutes	12	3	0	12 (80%)	3 (20%)	15 (100%)
Loose Buffalo milk	200 minutes	14	1	0	14 (93.3%)	1 (6.6%)	15 (100%)
Fresh Loose milk	200 minutes	0	30	0	0 (0%)	30 (100%)	30 (100%)
Total		0	77	13	43 (47.7%)	47 (52.2%)	90 (100%)

Note: Values in parentheses represent percentage

Table 3.1: MBRT for milk

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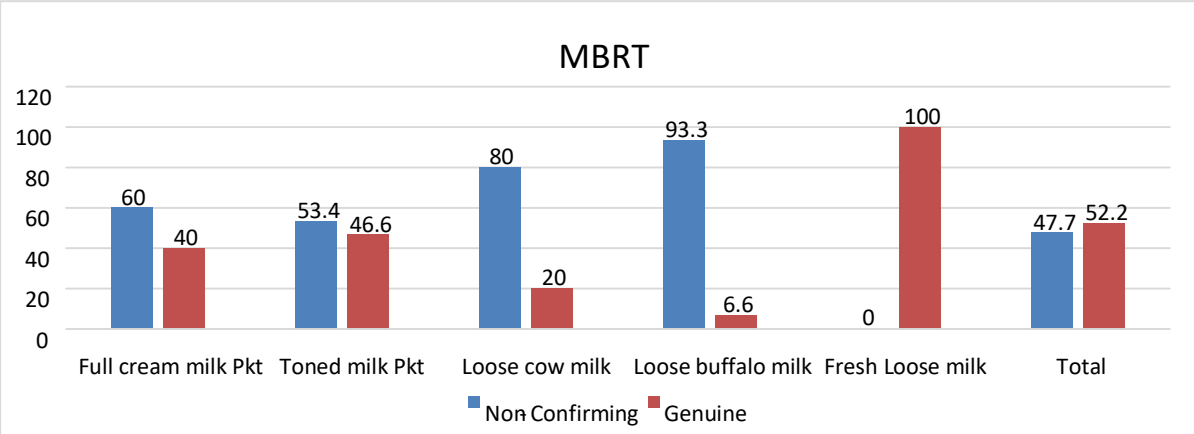

Fig 3.1: MBRT results for milk samples

Type of milk	Source	Region	Phosphatase		Standard Requirement
			Negative	Positive	
			Count	Count	
Fresh Loose	Buffalo	East	0	10	Positive
		North	0	10	
		South	0	10	
Loose	Buffalo	East	0	5	Positive
		North	0	5	
		South	0	5	
	Cow	East	0	5	Positive
		North	0	5	
		South	0	5	
Poly Packed	FCM	East	5	0	Negative
		North	5	0	
		South	5	0	
	TM	East	5	0	Negative
		North	5	0	
		South	5	0	

Table 3.2: Phosphatase Test

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3.2. Phosphatase Test

Alkaline phosphatase (ALP) is an enzyme present naturally in raw milk and is considered to be an indicator of proper milk pasteurization. Pasteurization is a process of heating milk at 63°C and holding it at this temperature for 30 minutes or heating milk at 71.5°C and holding it at this temperature for at least 15 seconds. Complete pasteurization inactivates the enzyme to levels that are undetectable by conventional methods. However, the failure to detect ALP activity does not guarantee that the milk is pathogen-free. The results of ALP activity tested on 90 milk samples collected from different zones are shown in Table 3.2. As it is depicted by the Table 3.2, all the samples i.e. both packaged and loose milk samples met the prescribed standards. All the 60 loose milk samples tested were positive for the ALP activity. All the 30 packaged milk samples which were pasteurized were negative for ALP enzyme activity indicating proper pasteurization of the milk samples. Similar results were obtained by other researchers **[26-28]**.

3.3. Coliform Test for milk

Presence of coliforms is an indicator of faecal contamination and thus the possibility of the presence of pathogens in the food and water samples. The coliform count is the number of coliforms in a sample that grow and form distinctive countable colonies on *Violet Red Bile Agar* after incubation at 37°C (98.6°F) for 24 hours. Coliforms are generally present in food that has been faecally or environmentally contaminated. The Coliform count in milk should be less than 10 according to FSSAI, 2011.

On analyzing 90 milk samples for presence of coliforms, it was found that 38 samples (42%) had higher number of coliform than the standards laid down by FSSAI (Table 3.3). All the 30 (100%) loose milk samples supplied by the vendors had coliforms counts >10. All the 30 (100%) fresh milk samples collected at source showed the absence of coliforms. Surprisingly coliforms were also detected in 8 (26.7%) of the 30 packaged milk samples that always claims to be free from coliforms (Figure 3.2). Similar results were reported for loose milk by other reporters **[23, 29-33]**. Others also reported higher level of coliform in packaged milk **[34]**. Presence of coliform in packaged milk indicates some kind of leakage and low maintenance of milk plant. Overall results indicated that at source milk, was free from coliform but before reaching to the consumers, it was contaminated which may be due to the unhygienic conditions and practices followed by the milk producers.

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Type of Milk	Milk Coliform (standard)	<10	≥ 10	Non-Confirming	Genuine	Total
Full cream	<10	12	3	3 (20%)	12 (80%)	15 (100%)
Toned	<10	10	5	5 (33.3%)	10 (66.6%)	15 (100%)
Loose cow milk	<10	0	15	15 (100%)	0 (0%)	15 (100%)
Loose Buffalo milk	<10	0	15	15 (100%)	0 (0%)	15 (100%)
Fresh Loose milk	<10	30	0	0 (0%)	30 (100%)	30 (100%)
Total		22	68	38 (42.2%)	52 (57.7%)	90 (100%)

Note: Values in parentheses represent percentage

Table 3.3: Coliforms in milk

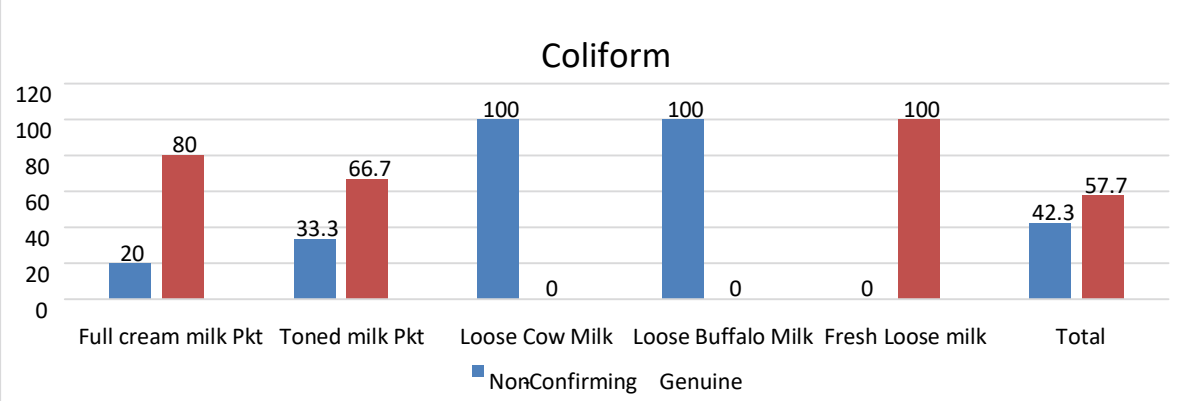

Fig 3.2: Coliforms in milk

3.4. SPC (Standard Plate Count) Test

The Standard plate count is a measure of bacterial activity in milk. SPC determines the total number of bacteria, fungi and yeasts that grows under aerobic conditions. As per FSSAI, 2011, it should not be more than 50,000 CFU/gram at plant level. It must be noted that this requirement is only at plant level but the consumer receives milk at retailer level. A high level of SPC was found in loose milk samples. About 26 of 30 (86.6%) loose milk samples supplied by the vendors had SPC >2, 00,000 and thus were substandard (Table 3.4).

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Other researchers also reported similar results **[23, 29, 30, 32 and 33]**. A high level of microbial count in milk may be due to non- maintenance of cold chain (below 8°C) during storage and transportation, use of contaminated water for adulteration or unhygienic milking conditions.

Of the 30 packaged milk samples, only two were having microbial count (50,000-2,00,000) above prescribed level while rest 28, were falling within the prescribed standard count of 30,000-50,000. Surprisingly, all the at source fresh loose milk samples, were of high quality and had bacterial count within prescribed limit (Figure 3.3). It is advisable for the consumers not to purchase packaged milk kept in open crates as it increases the chance of microbial growth. It must be mentioned here that the researcher had collected refrigerated packaged milk samples during early morning hours for the present study.

Type of Milk	**Milk SPC (required)**	**30,000-50,000**	**50,000-2,00,000**	**>2,00,000**	**Non-Confirming**	**Genuine**	**Total**
Full cream	30,000-50,000	14	1	0	**1 (6.6%)**	**14 (93.3%)**	**15 (100%)**
Toned	30,000-50,000	14	1	0	**1 (6.6%)**	**14 (93.3%)**	**15 (100%)**
Loose cow milk	2,00,000	2	1	12	**12 (80%)**	**3 (20%)**	**15 (100%)**
Loose Buffalo milk	2,00,000	1	0	14	**14 (93.3%)**	**1 (6.6%)**	**15 (100%)**
Fresh Loose milk	2,00,000	25	5	0	**0 (0%)**	**30 (100%)**	**30 (100%)**
Total		**56**	**8**	**26**	**28 (31.1%)**	**62 (68.8%)**	**90 (100%)**

Note: Values in parentheses represent percentage

Table 3.4: SPC in Milk

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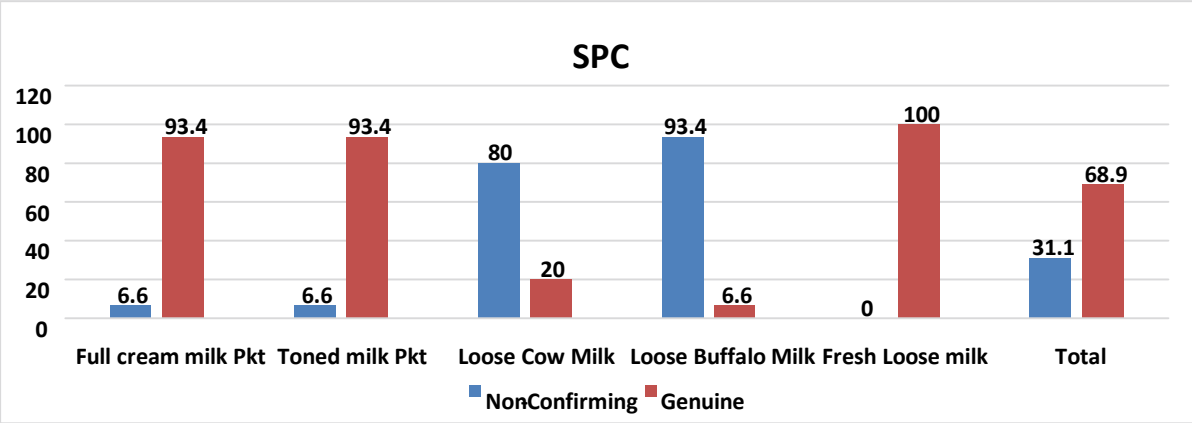

Fig 3.3: SPC in milk

4. Conclusions

Microbial analysis of milk detected coliforms in few packaged milk samples. This may be due to low maintenance and leakage in milk plants. All the vendor milk samples collected (100 percent) again failed the microbial quality test. Surprisingly, at source all the milk samples collected were of high quality i.e. having coliforms less than the prescribed limit (<10). A high level of microbial count (SPC) was found in raw milk samples collected from vendors and thus, were substandard (above 2, 00,000 CFU/ml). It might be due to non- maintenance of cold chain during storage and transportation (below 8 degree Celsius). From the results obtained, it can be concluded that fresh milk at source was of very good quality and was meeting all the standards laid down by FSSAI for its compositional and microbial quality. However, loose milk quality deteriorated when it reached the consumer. Quality of packaged milk was marginally affected due to non-maintenance of cold chain. Overall, half of the samples were falling below the prescribed standards. Maximum deviation was observed in loose vendor milk whereas packaged milk was marginally below the standards.

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