MICROBIOLOGICAL EVALUATION OF RAW AND PASTEURIZED MILK FROM MALWA REGION OF PUNJAB, INDIA

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Abstract
The present study was conducted to analyze the microbial (total viable bacteria, total coliforms, and total fungal) quality, and physiological properties of the raw and pasteurized Milk in the Malwa region of Punjab, India. We collected twenty milk samples (ten each from Raw and pasteurized vendor) in and around Bathinda district of Punjab. Milk quality was initially checked by methylene blue reduction test (MBRT), and Clot on Boiling (COB)test. These samples were used for quantification of bacterial contaminants by standard plate count method on selective media such VRBA, EMBA, EDAB, MacConkey Agar, and Potato Dextrose Agar to identify the various microorganisms. Total viable Count were high in milk sample which ranges from $4.3X10^6$ to $>3.0X10^7$ CFU/ml and total fungus number ranges from 1.1×10^5 to $>3\times10^7$ cfu/ml.Using biochemical methods, we further confirmed the identity of bacteria like Escherichia coli, Salmonella, and Enterobacter aerogenes. The current study reveals that both raw and industrially processed milk sample does not conform to microbiological standard and may not be safe to consumption without boiling. There is an urgent need to maintain proper hygiene and strict regulation while processing the milk in industrial plant and local supplier.

Keyword: Bacterial count, Biochemical identification, Coliform Bacteria, Contamination of Milk, Milk borne diseases, Pasteurization.

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1. INTRODUCTION

Milk is regarded as a capricious biological fluid due to it changing nature from one species to other interspecies, different breeding types, health and nutritional status of animal, the stages of lactation, their age, and time gap between milking(Fox and McSweeney, 1998). Milk has its own neutral flavor due to presence of lactose, salts, proteins, and fats. The mild sweet taste of milk is due to presence of lactose and presence of fat gives a rich mouthfeel in whole milk (Stephanie Clark et al., 2009).
Milk is an excellent growth medium for many microorganisms. Bacterial contamination of unpasteurized milk arises from various sources such as water, air, soil, feed, milking equipment, and feces. Moreover, variation in housing strategies and feeding of milch animals may affect the microbial quality of milk. The storage and transport of refrigerated raw milk leads to change in microbial population from gram-positive to gram-negative organisms which account for more than 90% of bacterial cells. Pasteurization of raw milk is effective in eliminating the microorganisms but thermoduric microbes of the genera Microbacterium, Micrococcus, Streptococcus, Lactobacillus, Bacillus, Clostridium, and occasionally some gram-negative bacillican sustain high temperatures (Fitzstevens et al., 2017, Quigley et al., 2013).Milk is often contaminated with pathogenic microorganisms such as Escherichia coli, Clostridium, Listeria monocytogenes, Salmonella, and Streptococcus and major cause of transmission of disease. The contaminants also include yeasts and molds which play a significant role in spoilage before pasteurization(Sørhaug, 2011). These are usually introduced into the milk as

contaminations during the milking procedures or as recontaminations after the heat treatment. As mentioned in the prevention of food adulteration (PFA) rules, India(1956), Milk borne diseases are commonly associated with species such as Salmonella spp., Enterococcus, and E. coli O157: H7 infection (Koushki et al., 2016). Moreover, fungal intoxication, caused by *Aspergillus flavus*, is the major cause of aflatoxins in contaminated milk (Nahidul-Islam et al., 2018).

Examination for the presence and enumeration of specific micro-organisms is an integral part of quality assurance plans in food industry. Herein, we have collected raw and pasteurized milk samples from Malwa region of Punjab, India **(Table 1)**. We first performed the initial quality check for acidity using clot on boiling assay, and microbial quality using methylene blue reduction assay. Later, we analyzed the milk samples for their microbial content using total viable count, total coliforms count, and total fungal count. We then used selective and differential media to determine the total number of Escherichia coli, Salmonella, and Enterobacter species present in the milk samples. Finally, we characterized and confirmed the isolated colonies using biochemical test.

Material and Methods

Sample collection --The study was conducted at Bathinda district, Punjab, India. A total twenty milk samples of ten different pasteurized (branded) and ten samples of local milk supplier (raw)were collected (M1 to M20) (**See Table 1**). All the possible precautions were taken to avoid external contamination at the time of collection and during processing of Milk. Samples were stored at 4^0C and sterile conditions were maintained throughout the analysis. Sample, collected in month of January to February, was stored in propylene tube and stored immediately at 4^0C. Initial experiments for quality control and microbial enumeration were performed within 72 h.

Clot on Boiling (COB) and Methylene Blue Reductase Test–These tests were performed as per guideline provided by Food Safety and Standards Authority of India for calculating the Milk Quality in the food samples (India, Version-IX (29.03.2019)).

Enumeration of Microbial Count- We employed spread plate technique after serially dilutingthe milk sample by following standard protocol (APHA, 1976; FDA, 2001). Plate Count Agar, MacConkey Agar, Potato Dextrose Agar were used for the growth of Total Viable bacteria (TVC), Total Coliform Bacteria (TCB) (Lactose fermenting and Non-lactose fermenting), and Total Fungus count (TFC) respectively(Pappa et al., 2019). Media such as VRBA, EMBA, and EDAB was used to determine species level identification of Escherichia coli, Salmonella, and Enterococcus species. The colonies appear after 37^0C was counted manually, andactual numbers of bacteria were estimated ascolony forming unit

ID	Source	Type
M1.	University campus vendor I	Raw
M2	Saras pasteurized milk	Pasteurized
M3.	DAIRY RATAN pasteurized milk	Pasteurized
M4.	VERKA full cream pasteurized milk	Pasteurized
M5.	University campus vendor II	Raw
M6.	TIME FRESH pasteurized milk	Pasteurized
M7.	Tirath standard milk	Pasteurized
M8.	BABA full cream pasteurized milk	Pasteurized
M9.	KOHLI soya flavored milk	Pasteurized
M10.	Local vendor (Bathinda village)	Raw
M11.	Tungwali Village sample	Raw
M12.	Punjab dairy (university campus)	Raw
M13.	Local dairy, Bathinda	Raw
M14.	Cow's fresh milk (Sukha village)	Raw
M15.	Buffalo's fresh milk (Sukha village)	Raw
M16.	Mother dairy lite	Pasteurized
M17.	Buffalo's fresh milk Bagha village	Raw
M18.	Nasibpura Village	Raw
M19.	SUKHAD pasteurized milk	Pasteurized
M20.	TODAY'S MILK pasteurized milk	Pasteurized

Table 1: Source of Milk samples used in this study- Milk samples, collected from either Milkman (Raw) or packaged pasteurized (highlighted), was transferred aseptically into fifty milliliter polypropylene tube and stored immediately at 4^0C. Initial experiment were performed within a week of collection.

(CFU/ml).

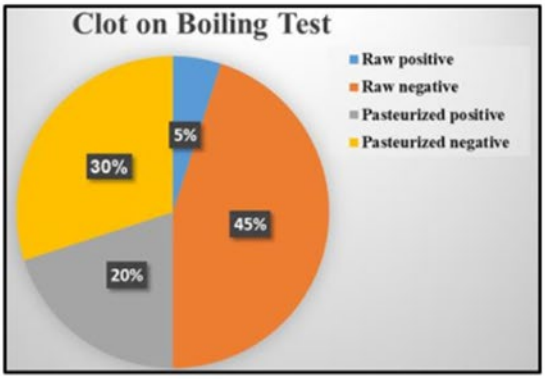
Biochemical identification – For species level identification, individual bacteria collected from Violet Red Bile agar (VBRA) were examined for standard biochemical test such as IMVIC, Vogues-Proskauer, and Citrate utilization using the standard protocol as described previously (American Society of Microbiology, 2015).

Result and Discussion

Preliminary testing of Milk

After collection, we immediately performed Clot of Boiling (COB) test to measure stability of milk for heat processing. Out of twenty samples, five (25%) showed positive results **(Figure 1A and 1B)**. Amongst the ten raw samples, one (10%) was positive and amongst the pasteurized ten samples four (40%) showed positive results. Positive test indicates acidity generally above 0.17% which is not suitable for consumption and has tendency spoiled readily. Although not reliable, Methylene Blue reduction test (MBRT) is a fast method to detect microbial contamination in dairy product. MBRT experiments showed that out of twenty samples, three were poor, five were average, ten were good, and two were excellent **(Figure 1A and 1C)**. Out of ten samples of pasteurized milk, 10% was poor, 10% was average, 60% were good, and 20% were excellent **(Figure 1A).** To summarize, our data suggest 40% pasteurized milk has higher acid content while raw milk samples has higher number of microbial contaminations.

Sample ID	Clot on Boiling (COB) test	MBRT Decolourization Time (h)	Class
M1	Positive	6	Good
M2*	Negative	>6	Excellent
M3*	Positive	6	Good
M4*	Negative	6	Good
M5	Negative	6	Good
M6*	Positive	6	Good
M7*	Negative	2	Poor
M8*	Positive	6	Good
M9*	Negative	6	Good
M10	Negative	3	Average
M11	Negative	6	Good
M12	Negative	3	Average
M13	Negative	2	Poor
M14	Negative	4	Average
M15	Negative	1	Poor
M16*	Negative	>6	Excellent
M17	Negative	4	Average
M18	Negative	5	Good
M19*	Positive	4	Average
M20*	Negative	6	Good



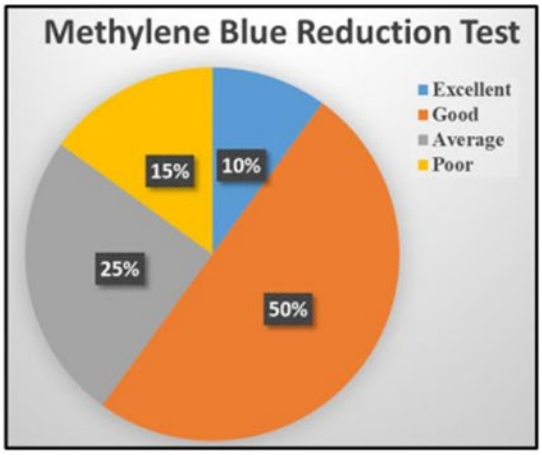


Figure 1: Preliminary Characterization of Milk Samples- Raw and pasteurized milk samples were initially tested for their acidity content, and microbial quality test using Clot on boiling test (coagulation/COB) and Methylene Blue Reduction Test (MBRT) respectively. Most of the non-pasteurized samples, and 40% pasteurized milk samples failed COB test.(* represent pasteurized milk).

Total Viable Count of Bacteria – Previous studies suggested that MBRT may not in cognizance with total microbial count so we next plated the diluted milk samples (10^{-5}) on Plate Count Agar (PCA) and counted the number of colonies that appear after 24 h of incubation at 37^0C. The total viable count of Raw milk samples ranges from $4.8X10^6$ to $>3X10^7$**(Figure 2, left panel)**while total viable count in pasteurized milk varies from $4.3X10^6$ to more than $3X10^7$**(Figure 2, Right panel)**. These data suggest that both raw and pasteurized milk sample in Malwa region has way too high number of bacterialcounts which is beyond safe limit as per FSAAI standard.

Total Coliform Count- Coliform, gram-negative, non-spore forming aerobic or facultative anaerobic bacilli, are important indicator for unhygienic condition prevailing during the milk handling(Martin et al., 2016). To determine the number of lactose fermenting and non-lactose fermenting bacteria, we plated diluted milk samples on MacConkey Agar and count the number of pink and while colonies that appear after 48 h of incubation at 35^0C.

Table 2 shows the total number and percentage of the lactose fermenter and non-lactose fermenter on MacConkey agar. All except M9 (Flavored soya milk) consist of both lactose fermenter and non-lactose fermenting bacteria. Moreover, 80% of raw milk sample consist of Non-lactose fermenting bacteria while only 30% pasteurized milk sample show the presence of Non-lactose fermenter **(Table 2).** In summary, high number of coliforms (e.g., $>10^3$ CFU/mL) in raw milk, and pasteurized may indicates harmful practices on the farm, inadequate refrigeration, and presence of coliform mastitis in Malwa region of Punjab, India.

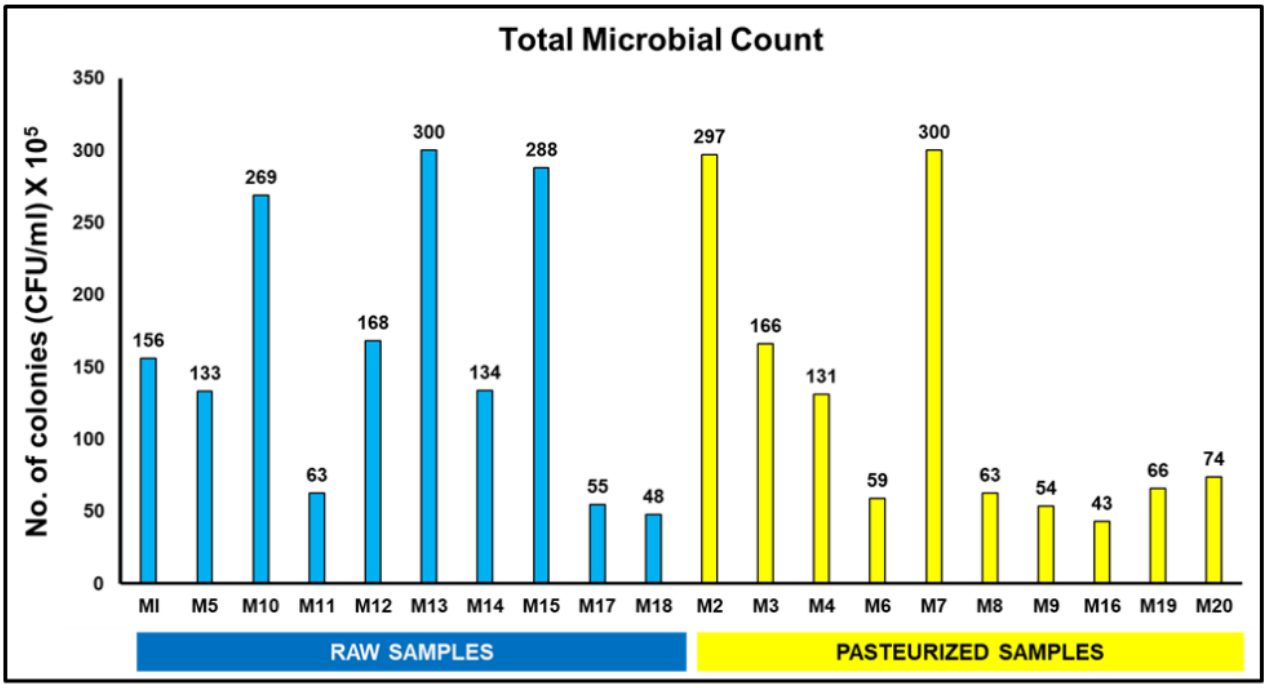


Figure 2 : Analysis of Total Viable Count – Plate Count Agar (PDA) assay was used to determine the bacterial load in Milk samples. Milk sample (both raw and pasteurized) was serially diluted in water and one ml of diluted samples (10^{-5}) was plated on freshly prepared media. Number of colonies was counted after 48 h of incubation at 37^0C. Colonies more than 250 is considered as too numerous too count.

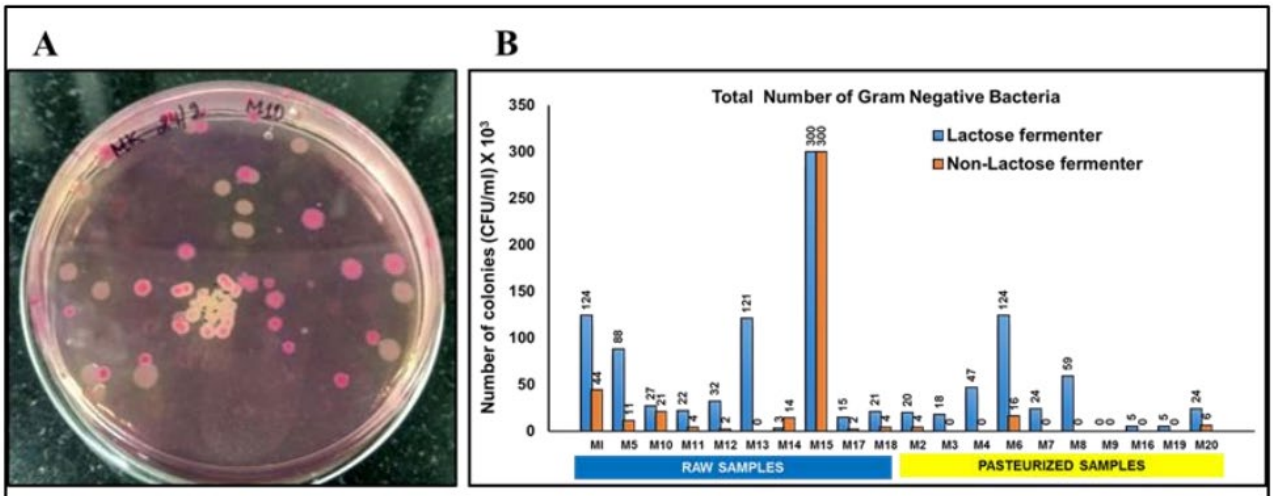
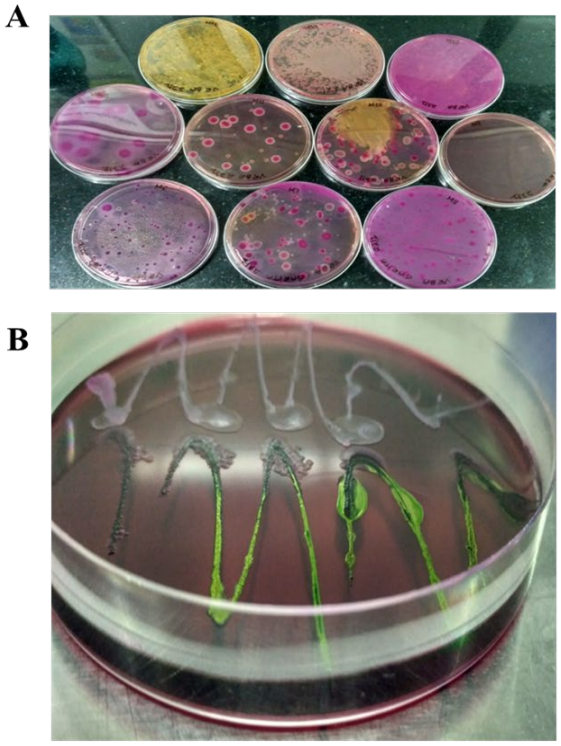


Figure 3 : Analysis of Lactose and Non-lactose fermenting bacteria – MacConkey Agar plating was performed to differentiate between lactose fermenting, and non-fermenter microbes. Milk sample (both raw and pasteurized), serially diluted in sterile water,(10^{-3}), was spread on Petri plate. Number of pink colonies and white colonies was counted after 36 h of incubation at 37^0C. Colonies more than 250 is considered as Too Numerous Too Count (TNTC). **(A)** Representative plate showing pink colonies of Lactose Fermenting Bacteria and colorless or transparent colonies of Non-lactose fermenting Bacteria **(B)** Graphical data showing number of lactose fermenter and non-lactose fermenter in milk.



C

ID	*Enterobacter* (CFU x 10^3)	*Escherichia coli* (CFU x 10^3)	*Salmonella* (CFU x 10^3)
M1	0	0	212
M2*	4	0	0
M3*	0	0	118
M4*	25	6	9
M5	0	50	0
M6*	0	6	6
M7*	40	6	0
M8*	36	14	0
M9*	0	0	0
M10	60	9	46
M11	50	14	-
M12	19	1	-
M13	TNTC	TNTC	-
M14	-	TNTC	TNTC
M15	TNTC	TNTC	-
M16*	-	2	-
M17	-	11	-
M18	-	2	-
M19*	6	2	-
M20*	8	-	-

Figure 4 : Presumptive and confirmatory test for number of coliform (A) Petri plates showing pinkish red with bile ppt. colonies of *Escherichia coli*, pinkish red colonies of *Enterobacter* and orangish yellow or colorless colonies of *Salmonella* on Violet Red Bile Agar (VRBA) **(B)** Petri plates showing confirmatory test for Escherichia coli in Green Metallic Sheen and *Salmonella* colorless colonies on Eosin Methylene Blue (EMB) Agar **(C)** Table showing total number of Enterobacter, Escherichia coli and Salmonella species (* denotes pasteurized milk)

Presumptive and Confirmatory determination for Coliform

In order to determine the number of gram-negative coliform, we further spread diluted milk sample on Violet Red Bile Agar (VBRA) media which is a presumptive test to calculate the number of coli-aerogenes microbes present in water, milk, and other dairy food products. Amongst the coliforms, E.coli was dominant in all the samples **(Figure 3A and 3C)**. Out of twenty samples, nineteen samples showed the presence of coliforms. We also performed confirmatory test for E.coli and Salmonella species by streaking the colonies on Eosin Methylene Blue (EMB) agar **(Figure 3B)**. To confirm the identity of bacterial species,we isolated thirty-two colonies from nineteen samples and characterized them as Escherichia coli, Enterobacter, and Salmonella on the basis of biochemical tests. Our biochemical test confirmed the presence of E. coli (40.6%), Enterobacter (28%), Salmonella (12.5 %), and unidentified (18.7%) among all the 32 isolates. In raw samples, E.coli was 41%, Enterobacter was 29.4%, Salmonella was 5.8% and 23.5 % were unidentified from 17 isolates. In pasteurized samples, E.coli was 40%, Enterobacter was 26.6%, and Salmonella was 20% and 13.3 % were unidentified from 15 isolates **(Table 4)**. In summary, using presumptive, completed, and biochemical test, we showed the existence of *Escherichia coli*, Enterobacter, and Salmonella in both raw and pasteurized milk.

Enterococcus species are leading cause of urinary tract infections, bacteremia, and endocarditis. Infections due to *Enterococcus faecalis* are more virulent in comparison to *Enterococcus faecium* so we further analyzed the total number of Enterococcus species after plating the diluted milk samples on Enterococcus Differential Agar Base (EDAB). Out of 20 samples, 18 showed the presence of Enterococcus spp. **(Table 4)** and Enterococcus colonies did not appear in two (M16 and M19). The percentage of isolates on EDAB were; E. faecium 40% and E. faecalis 30%, both 20% and none 10%. In raw samples, the minimum number was 1.7 x 10^3 CFU/ml. The minimum number in pasteurized samples was 0.5 x 10^3 CFU/ml. **(Figure 4)**. These data suggest the presence of deadly pathogenic bacteria in raw and pasteurized milk.

Samples	Total Bacteria (CFU x 10^3)	Lactose fermenting (CFU x 10^3)	% of Lactose fermenter (Out of 100)	Non-Lactose fermenting (CFU x 10^3)	% of Non-Lactose fermenter (Out of 100)
M1	168	124	73.8	44	26.2
M2*	24	20	83.33	4	16.66
M3*	18	18	100	-	0
M4*	47	47	100	-	0
M5	99	88	88.89	11	11.11
M6*	140	124	88.57	16	11.43
M7*	24	24	100	-	0
M8*	59	59	100	-	0
M9*	-	-	-	-	-
M10	48	27	56.25	21	43.75
M11	26	22	84.62	4	15.38
M12	34	32	94.11	2	5.89
M13	121	121	100	-	0
M14	17	3	17.65	14	82.35
M15	TNTC	TNTC	TNTC	TNTC	TNTC
M16*	5	5	100	-	0
M17	17	15	88.23	2	11.76
M18	25	21	84	4	16
M19*	5	5	100	-	0
M20*	30	24	80	6	20

Table 2 : Data showing total number and percentage of Lactose and Non-lactose fermenter microbes in Milk. All except M9 samples were positive both for Lactose fermenting and Non-lactose fermenting bacteria. The number of gram negative bacteria are beyond the permissible limit

Samples	*Enterococcus faecalis* (CFU x 10^3)	*Enterococcus faecium* (CFU x 10^3)
M1	-	TNTC
M2*	-	202
M3*	-	97
M4*	-	154
M5	-	188
M6*	9	63
M7*	147	23
M8*	267	209
M9*	33	-
M10	159	TNTC
M11	-	31
M12	55	66
M13	212	-
M14	2	-
M15	TNTC	TNTC
M16*	-	-
M17	2	-
M18	-	5
M19*	-	-
M20*	-	6

Table 3: Enumeration of Enterococcus bacteria - Diluted Milk samples (10^{-3}) were plated on Enterococcus differential agar base **(EDAB)** to identify the number of *Enterococcus faecalis and Enterococcus faecium* present in the milk.

Organism	Sample ID	Indole	Methyl red	Vogues-Proskauer	Citrate	Catalase	Oxidase
Escherichia coli	M5, M6, M7, M8, M10, M13, M15, M16, M17, M18, M19.	Yes	Yes	No	No	Yes	No
Enterobacter aerogenes	M4, M10, M11, M12, M13, M14, M19, M20.	No	No	Yes	Yes	Yes	No
Salmonella	M1, M3, M4, M6, M12.	No	Yes	No	No	Yes	No

Table 4: Species level confirmation of bacteria – Individual colony, collected from VBRA agar plates, were tested by biochemical methods such as IMVIC test, catalase, and oxidase test and presence of *Escherichia coli*, *Enterobacter aerogenes* and *Salmonella* species was confirmed.

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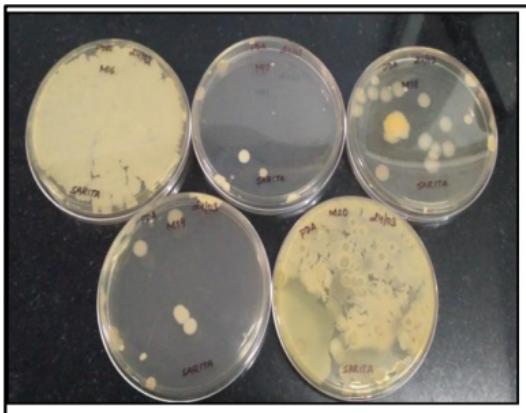

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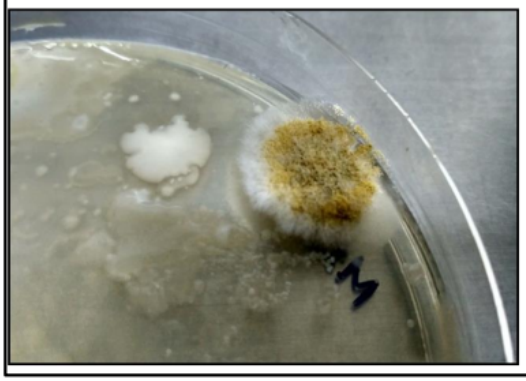

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ID	Day 1 (Candida albicans) (CFU x 10^5)	Day 5 (Fusarium spp.) (CFU x 10^5)	Day 7 (Aspergillus spp.) (CFU x 10^5)
M1	83	19	0
M2*	27	0	0
M3*	49	17	0
M4*	25	0	0
M5	91	14	0
M6*	TNTC	0	0
M7*	69	3	0
M8*	15	15	0
M9*	18	4	0
M10	TNTC	4	0
M11	44	6	0
M12	36	3	0
M13	155	0	1
M14	16	2	0
M15	TNTC	0	0
M16*	1	0	0
M17	6	0	0
M18	20	0	1
M19*	7	0	0
M20*	24	0	1

Figure 5: Enumeration of Total Fungus Count in Milk (A) Petri plates showing *Candida albicans* on potato dextrose agar after 24 h of incubation **(B)** Petri plates showing presence of *Aspergillus* species in Milk **(C)** Table showing total number of *Candida albicans, Fusarium* species, and *Aspergillus* species after day one, day five, and day seven from diluted milk samples (10^{-5}) (*denotes pasteurized milk).

Estimation of Total Fungus Count

Presence of Fungi has tremendous spoilage potential in Milk industry since mold such as Aspergillus species produces deadly mycotoxin. To determine the total fungal count, we plated the diluted samples (10^{-5}) on Potato Dextrose Agar (PDA). All the samples showed the presence of Candida albicans on day one **(Figure 5A)**. We also observed Fusarium colonies on day five in six raw and four pasteurized milk samples. On day seven, Aspergillus spp. **(Figure 5B)** were obtained in two raw samples (M13, M18) and one pasteurized sample (M20).The range of total fungal count was 1.1×10^5 to 4.8×10^7cfu/ml in raw and pasteurized milk. Values of yeast and mold count recorded in the milk sample M16 was minimum and maximum in M6, M10, and M15. The respective values recorded were $0.1X10^6$ - $3X10^7$ CFU/ml**(Figure 4C)**. This result clearly showed the insanitary conditions exist during the handling and sale of milk in Malwa region of Punjab.

4. CONCLUSION

This investigation was taken up to study the quality of raw and pasteurized milk microbiologically as well as biochemical identification of disease associated bacteria in Malwa region of Punjab, India. In India, Food Safety and Standards Authority of India(FSSAI)are the prime government regulatory authorities which determine the quality of food product. In a recently conducted nationwide survey by FSSAI show that 93% milk samples (out of 6432 samples) were safe for human consumption. In their survey, they only collected six samples from

Bathindadistrict (raw and pasteurized status not mention clearly) and tested from the presence of food adulterant such as urea and presence of aflatoxin M1 level(Food Safety and Standards Authority of India, 2018).In this paper, we first determine the microbiological quality of Milk and later enumerate the presence of disease-causing non-lactose fermenter present in the milk samples. Preliminary analysis using MBRT and clot on boiling assay clearly demonstrate the poor quality of Milk supply in this region. Most of the raw and pasteurized milk samples collected from different locations of Bathinda showed poor level of hygiene since total viable count, total number of enteric bacteria, and total fungal count exceeded permissible limit(GOEL)and confirm the previous report of presence of E. coli in milk in the Punjab region(Agarwal A, 2012). Using differential media, our studies also reveal the presence unacceptable numberof *Enterococcus faecalis* and *Salmonella species* in the Milk samples. Unhygienic milking, its handling, failure in maintaining cold chain during transportation, contamination after pasteurization, personal, and utensil hygiene and contaminated adulterated ingredients such as poor quality of water are the main factors responsible for the microbial deterioration of the milk. Our studies clearly show the poor quality of both raw and pasteurized milk in the Malwa region of Punjab. We concluded that the samples M2, M7 and M13 were of very poor quality, M9 was good (Kohli Soy Flavored Milk) and rest of them were fair. The gap between demand and supply is one of the important reasons for the milk adulteration. Based on our finding, there is an urgent need to take strict measures against companies or individuals for selling poor microbial quality milk. A combination of regular monitoring, proper maintenance, use of safe water, and proper training of workers about health and hygiene could be appropriate choice to develop the quality of Milk. In summary, there is needto create consumer awareness regarding the microbiological quality of milk.

Author contributions: Sarita and M.V.K. collected, designed, and executed experimental work. PG analyzed the data and wrote the first draft. SB supervised and finalizes manuscript. All authors read and approved the final manuscript.

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Competing Interests: The authors declare that they have no competing interests.

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