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Bacteria From Boiled And Un-boiled Pasteurized Milk: A Comparative Analysis Of Microbiological And Biochemical Properties

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ABSTRACT

Milk is one of the most common nutritious food consumed by most of the Indians. But we Indians mainly consume milk, even packaged milk, after proper boiling. As pasteurization is not a full sterilization process, number of bacteria after pasteurization should be reduced but not eliminated. This paper is mainly focused on the characterization of microorganisms isolated from Pasteurized and boiled Pasteurized milk samples. The prevalence, isolation, and biochemical characterization of different bacteria from both milk samples were studied. The milk samples were used for bacterial colony isolation using standard microbiological methods. The isolated colonies were tested for their Gram character, endospore formation and tests for catalase, oxidase, IMViC, urease, litmus, triple sugar fermentation & hemolytic activities.

Keywords: *Pasteurized milk, boiled milk, biochemical activities*

Introduction

Since ancient ages milk and milk products have been used by the human population. During the past few decades, with regard to living standards, diet, hygiene and usage of antibiotics, developing countries have been changing fast. Society considers the emotional and cultural importance of milk, the very first food of mammals including humans. Milk has been considered as nature's most perfect food for humans since they have been habituated with it since birth. An important constituent of a balanced diet for human beings includes milk and dairy products, which have long been recognized as these products provide a wide range of essential nutrients.

Milk and milk products serve as a prime habitat to an immense variety of microorganisms and their presence in milk leads to the large-scale variations in attributes of milk like odor, taste and quality of milk. Adulteration in milk and dairy products by the presence of microorganisms arises from inside of udder, outside of the udder, by vendors and equipment which is used for storage to transport to long distances. In the udder of a mammal, milk is a sterile substance but as soon as milk passes out of the teat of the cow, it is inoculated by normal flora of the animal. The origin of pathogenic microorganisms present in milk may be from a cow, or it may be in humans, and in some cases it may be transmitted by both.

Pathogenic bacteria in raw milk are also involved in the spoilage of milk and these pathogens are predominantly resulting from unclean conditions and poor handling. These pathogenic microorganisms in raw milk are responsible for many milk-borne illnesses like brucellosis, typhoid fever and tuberculosis especially in developing countries.

Drinking milk arises most of the milk-borne disorders that are caused by *Listeria monocytogenes, Salmonella sp., Campylobacter sp., Staphylococcus aureus, Yersinia sp., Escherichia coli* and *Clostridium botulinum.*

Boiling of milk in developing countries is a common practice and it increases the shelf life of milk as well as enhances the taste of the milk. [1] reported that when raw milk was exposed to boiling for 15 seconds, there is a noticeable decrease in total bacterial load. In colloidal forms in milk, lipids and proteins have a shielding role in microorganisms against heat treatments given to kill microorganisms, consequently some microorganisms are expected to survive in milk even after boiling.

Pasteurization is defined as a thermal process used to reduce health risks from pathogenic microorganisms present in milk and also to lengthen shelf life of the product. To treat different cases of mastitis various antibiotics are used. To cure diseases of cattle, antibiotics are usually used. The excessive use of these antibiotics has made antibiotic treatment ineffective and has managed to develop multiple antibiotic resistances among microorganisms causing diseases. Milk serves as an efficient vehicle for transmission of diseases to humans and approximately 90% of dairy-related diseases in human beings arise from unhygienic milk products. There are regulations that require proper hygienic handling of milk and its Pasteurization to protect public health against milk-borne infections.

Material and Methods

Isolation of bacterial colony

20 ml milk sample was equally divided into two parts in sterile test tubes each containing 10 ml.

One part of the sample (10 ml) was boiled for 15 minutes at 100° C to prepare boiled Pasteurized milk. Both boiled and un-boiled Pasteurized milk samples were serially diluted from 10^{-1} to $10^{-10}$and plated. The plates were then incubated at 37°C for 24 hours. After 24 hours the CFU value was then calculated. The single colonies formed in the spread plate were taken to prepare a pure culture of these microorganisms.

Staining

The pure cultures were taken for Gram staining to detect the Gram characteristics of the isolated microorganisms.
The pure cultures were taken for endospore staining also to ensure the presence of spore-forming bacteria in milk. 72 hours old pure cultures grown without glucose medium, were taken for this staining process in order to get more number of endospores.
The pure cultures were tested for catalase activity. This test was done in a clean, dry and grease-free glass slide by observing the evolution of oxygen bubbles. The organisms that showed the bubble formation is considered positive for the presence of catalase enzyme, that is catalase positive.
The pure cultures were used for the oxidase test. A small piece of filter paper was soaked in 1% Kovacs Oxidase reagent and dried. A well-isolated colony from a fresh bacterial plate was picked by using an inoculating loop and rub onto treated filter paper. Then observed for the colour changes.

Results

Isolation of bacterial colony

Single colonies isolated from Pasteurized milk sample:

10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
>300	>300	>300	>300	>300	278	260	212	150	89

Single colonies isolated from Boiled Pasteurized milk sample:

10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
>300	>300	>300	297	212	280	198	145	98	55

Colony morphology of isolated microorganisms:

Pasteurized milk sample:

Serial No.	Culture No.	Color	Colony Form	Elevation	Margin
1.	2(1)	White	Rhizoid/Root like	Flat	Entire(smooth)
2.	2(2)	White	Rhizoid/Root like	Flat	Entire(smooth)
3.	2(8)	White	Irregular	Flat	Lobate
4.	3(1)	White	Irregular	Raised	Lobate
5.	3(2)	White	Irregular	Umbonate	Undulated(Wavy)
6.	3(3)	White	Round	Pulvinate/Cushion like	Entire(Smooth)
7.	3(4)	Yellowish White	Punctiform	Convex	Entire(Smooth)
8.	3(7)	White	Irregular	Raised	Erose/serrated
9.	3(8)	White	Irregular	Raised	Lobate
10.	4(1)	Transparent White	Round	Flat	entire(smooth)
11.	4(2)	Orangish white	Round	Flat	entire(smooth)
12.	4(5)	Transparent White	Round	Flat	entire(smooth)
13.	4(6)	White	Punctiform	Convex	entire(smooth)
14.	4(7)	White	Round	Flat	entire(smooth)
15.	4(9)	White	Irregular	Convex	Lobate
16.	4(12)	White	Irregular	Flat	Lobate
17.	4(13)	Transparent White	Irregular	Flat	Undulated(Wavy)
18.	4(16)	White	Irregular	Flat	Curled
19.	4(17)	Transparent White	Irregular	Flat	Undulated(Wavy)
20.	4(20)	White	Filamentous	Flat	Undulated(Wavy)
21.	4(24)	White	Irregular	Convex	Curled
22.	5(3)	White	Irregular	Convex	Curled
23.	5(4)	White	Round	Pulvinate/Cushion like	Entire(smooth)
24.	5(5)	White	Irregular	Umbonate	Lobate
25.	5(6)	White	Irregular	Raised	Undulated(wavy)
26.	5(7)	White	Irregular	Umbonate	Erose/serrated
27.	5(8)	Off white	Irregular	Convex	Undulated(wavy)
28.	5(13)	White	Round	Convex	Entire(smooth)
29.	5(14)	White	Irregular	Convex	Lobate
30.	6(1)	White	Punctiform	Flat	Entire(smooth)
31.	6(2)	White	Irregular	Flat	Undulated(wavy)
32.	6(4)	White	Irregular	Pulvinate/Cushion like	Lobate
33.	6(6)	White	Punctiform	Convex	Entire(smooth)
34.	6(9)	Yellowish White	Round	Convex	Entire(smooth)
35.	6(10)	White	Irregular	Raised	Entire(smooth)

36.	6(11)	White	Round	Pulvinate/Cushion like	Entire(smooth)
37.	6(16)	White	Round	Convex	Entire(smooth)
38.	7(1)	White	Irregular	Pulvinate/Cushion like	Entire(smooth)
39.	7(2)	White	Irregular	Umbonate	Lobate
40.	7(3)	Transparent White	Irregular	Flat	Lobate
41.	7(6)	White	Irregular	Umbonate	Lobate
42.	8(2)	White	Irregular	Raised	Undulated(wavy)
43.	8(4)	White	Punctiform	Convex	Entire(smooth)
44.	8(5)	White	Round	Raised	Entire(smooth)
45.	8(6)	White	Round	Flat	Filamentous
46.	8(8)	White	Round	Raised	Entire(smooth)
47.	9(5)	White	Irregular	Convex	Entire(smooth)
48.	9(6)	Yellowish White	Round	Raised	Entire(smooth)
49.	10(2)	White	Irregular	Pulvinate/Cushion like	Lobate
50.	10(6)	White	Irregular	Umbonate	Lobate
51.	10(7)	White	Irregular	Umbonate	Lobate

(Fig: 1)

Boiled Pasteurized milk sample:

Serial No.	Culture No.	Color	Colony Form	Elevation	Margin
1.	P3	Transparent White	Round	Flat	Entire(Smooth)
2.	P7	Yellow	Round	Convex	Entire(Smooth)
3.	P9	White	Punctiform	Flat	Entire(Smooth)
4.	S1	Orange	Round	Flat	Entire(Smooth)
5.	S3	White	Round	Flat	Entire(Smooth)
6.	S5	White	Punctiform	Flat	Entire(Smooth)
7.	S13	White	Round	Convex	Curled
8.	S20	White	Round	Flat	Entire(Smooth)
9.	S23	White	Irregular	Convex	Undulated(Wavy)
10.	S25	Transparent White	Round	Flat	Entire(Smooth)
11.	S29	White	Round	Flat	Entire(Smooth)

(Fig: 2)

Staining

Gram Staining

Pasteurized milk sample:

Serial No.	Culture No.	Gram Characteristics	Shape
1. 1.	2(1)	Gram (–ve)	Short rod
2.	2(2)	Gram (–ve)	Short rod
3.	2(8)	Gram (–ve)	Rod
4.	3(1)	Gram (+ve)	Rod
5.	3(2)	Gram (+ve)	Rod
6.	3(3)	Gram (–ve)	Rod
7.	3(4)	Gram (–ve)	Rod
8.	3(7)	Gram (–ve)	Rod
9.	3(8)	Gram (+ve)	Rod
10.	4(1)	Gram (+ve)	Rod
11.	4(2)	Gram (+ve)	Rod
12.	4(5)	Gram (–ve)	Coccus
13.	4(6)	Gram (–ve)	Rod
14.	4(7)	Gram (+ve)	Rod
15.	4(9)	Gram (+ve)	Rod
16.	4(12)	Gram (+ve)	Rod
17.	4(13)	Gram (+ve)	Short Rod
18.	4(16)	Gram (+ve)	Rod
19.	4(17)	Gram (+ve)	Rod
20.	4(20)	Gram (+ve)	Short Rod
21.	4(24)	Gram (+ve)	Rod
22.	5(3)	Gram (+ve)	Rod
23.	5(4)	Gram (+ve)	Rod
24.	5(5)	Gram (+ve)	Rod
25.	5(6)	Gram (+ve)	Rod
26.	5(7)	Gram (-ve)	Rod
27.	5(8)	Gram (+ve)	Rod
28.	5(13)	Gram (+ve)	Coccus
29.	5(14)	Gram (+ve)	Rod
30.	6(1)	Gram (+ve)	Rod

31.	6(2)	Gram (+ve)	Rod
32.	6(4)	Gram (+ve)	Rod
33.	6(6)	Gram (+ve)	Rod
34.	6(9)	Gram (+ve)	Rod
35.	6(10)	Gram (+ve)	Rod
36.	6(11)	Gram (+ve)	Rod
37.	6(16)	Gram (-ve)	Short Rod
38.	7(1)	Gram (+ve)	Rod
39.	7(2)	Gram (+ve)	Rod
40.	7(3)	Gram (+ve)	Short Rod
41.	7(6)	Gram (+ve)	Rod
42.	8(2)	Gram (+ve)	Rod
43.	8(4)	Gram (+ve)	Rod
44.	8(5)	Gram (+ve)	Rod
45.	8(6)	Gram (+ve)	Rod
46.	8(8)	Gram (+ve)	Rod
47.	9(5)	Gram (+ve)	Rod
48.	9(6)	Gram (+ve)	Short Rod
49.	10(2)	Gram (+ve)	Rod
50.	10(6)	Gram (+ve)	Rod
51.	10(7)	Gram (+ve)	Rod

Boiled Pasteurized milk sample:

Serial No.	Culture No.	Gram Characteristics	Shape
1.	P3	Gram (+ve)	Short Rod
2.	P7	Gram (+ve)	Rod
3.	P9	Gram (+ve)	Coccus
4.	S1	Gram (+ve)	Coccus
5.	S3	Gram (+ve)	Coccus
6.	S5	Gram (-ve)	Rod
7.	S13	Gram (+ve)	Rod
8.	S20	Gram (+ve)	Rod
9.	S23	Gram (+ve)	Rod
10.	S25	Gram (+ve)	Rod
11.	S29	Gram (+ve)	Short Rod

(Fig: 3)

Endospore Staining:

Endospore Staining	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Positive Samples	2(8),3(1),3(2),3(3),3(4),3(7), 3(8),4(1),4(2),4(5),4(6),4(7), 4(9),4(12),4(13),4(16),4(17), 4(20),4(24),5(3),5(4),5(5),5(6), 5(7),5(8),5(13),5(14),6(1),6(2), 6(4),6(6),6(9),6(10),6(11),6(16), 7(1),7(2),7(3),7(6),8(2),8(4),8(6), 10(2),10(6),10(7)	P3,P7,S1,S3,S5,S13,S23,S25,S29
Negative Samples	2(1),2(2),8(5),8(8),9(5),9(6)	P9,S20

(Fig: 4)

Catalase Test:

Catalase Test	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Positive Samples	2(1),2(2),2(8),3(1),3(2),3(3),3(4),3(7), 3(8),4(2),4(5),4(6),4(7),4(9),4(12),4(13), 4(16),4(17),4(20),4(24),5(3),5(4),5(5), 5(6),5(7),5(8),5(13),5(14),6(1),6(2),6(6),	P3,P7,P9,S1,S3,S5,S13,S20,S25
	6(9),6(10),6(16),7(1),7(2),7(3),7(6),8(2), 8(4),8(5),8(6),8(8),9(5),9(6),10(2),10(6), 10(7)	
Negative Samples	4(1),6(4),6(11)	S23,S29

(Fig: 5)

Oxidase Test:

Oxidase Test	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Positive Samples	2(2),2(8),3(2),4(6),4(7),4(9),4(12), 4(16),4(17),4(20),5(4),5(5),5(6),5(7), 5(8),5(13),5(14),6(1),6(2),6(4),6(10), 6(11),7(6),8(2),8(4),8(5),8(6),8(8),9(6)	P9,S1,S3,S5,S13,S20,S23,S25,S29
Negative Samples	2(1),3(1),3(3),3(4),3(7),3(8),4(1),4(2), 4(5),4(13),4(24),5(3),6(6),6(9),6(16), 7(1),7(2),7(3),9(5),10(2),10(6),10(7)	P3,P7

(Fig: 6)

IMViC Test:

IMViC Test	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Positive Samples	Nil	Nil
Negative Samples	2(8),3(1),3(2),3(3),3(7),4(2),4(12), 4(13),4(16),4(24),5(3),5(4),5(5),5(7), 5(13),5(14),6(4),6(11),7(1),7(2),7(6), 9(5),10(2),10(6),10(7),2(1),2(2),3(4), 3(8),4(1),4(5),4(6),4(7),4(9),4(17),4(20), 5(6),5(8),6(1),6(2),6(6),6(9),6(10),6(16), 7(3),8(2),8(4),8(5),8(6),8(8),9(6)	P3,P7,P9,S1,S3,S5,S13,S20,S23,S25,S29

Haemolysis Test:

Haemolysis Test	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Positive Samples	2(8),3(1),3(2),3(3),3(7),4(2),4(12), 4(13),4(16),4(24),5(3),5(4),5(5),5(7), 5(13),5(14),6(4),6(11),7(1),7(2),7(6), 9(5),10(2),10(6),10(7)	P3
Negative Samples	2(1),2(2),3(4),3(8),4(1),4(5),4(6),4(7), 4(9),4(17),4(20),5(6),5(8),6(1),6(2),6(6), 6(9),6(10),6(16),7(3),8(2),8(4),8(5),8(6), 8(8),9(6)	P7,P9,S1,S3,S5,S13,S20,S23,S25,S29

Urease Test:

Urease Test	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Positive Samples	3(4),4(17),8(4),8(5),8(6),8(8)	S20
Negative Samples	2(1),2(2),2(8),3(1),3(2),3(3),3(7), 3(8),4(1),4(2),4(5),4(6),4(7),4(9), 4(12),4(13),4(16),4(20),4(24),5(3),	
	5(4)5(5),5(6),5(7),5(8),5(13),5(14), 6(1),6(2),6(4),6(6),6(9),6(10),6(11), 6(16),7(1),7(2),7(3),7(6),8(2),9(5), 9(6),10(2),10(6),10(7)	

Litmus Milk Test:

Litmus Test	Colour developed	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Acid Formation& Litmus Reduction	Pink Ring at top, White solution	4(6),4(7),6(1),	P9, S13
Alkali Formation	Blue ring at top, Grey solution	3(7),3(8),4(24),7(1),	S3, S5, S23, S25
Alkali Formation & Litmus Reduction	Blue ring at top, White solution	3(4),5(6),5(8),6(10),8(2),8(4), 8(5),8(8),9(5),9(6)	S20
Peptonization Reaction	Brown ring at top,Grey Solution	2(1),2(2),2(8),3(1),3(2),3(3), 4(2),4(5),4(9),4(12),4(16),4(20), 5(3),5(4),5(5),5(7),5(13),5(14), 6(2),6(4),6(6),6(9),6(11),6(16), 7(2),7(3),7(6),10(2),10(6),10(7)	
Curd Formation& Litmus Reduction	Grey ring at top, White solution	8(6)	
No Reaction	No change in colour	4(13),4(17)	S1, S29

Triple Sugar Iron Test:

Types of fermentation	Colour developed	Result obtained from Pasteurized milk sample:	Result obtained from Boiled Pasteurized milk sample:
Dextrose, Lactose, Sucrose fermentation + Acid Production	Yellow butt, yellow slant	3(1),3(2),3(3),3(7),4(2),4(5),4(7),4(9), 4(16),4(24),5(3),5(4),5(5),5(14),6(2),6(9), 6(11),6(16),7(1),7(2),10(2),10(6),10(7)	P3,P7,P9,S1
Only Dextrose fermentation + Acid Production	Yellow butt, Red slant	2(1),2(2),4(17),4(20),6(1),6(4),6(6),7(6), 8(5),8(6),8(8),9(6)	
No fermentation + Alkali Production	Red butt, Red slant	2(8),3(4),3(8),4(12),4(13),5(6),5(8),5(13), 6(10),7(3),8(2),8(4),9(5)	S3,S5,S13,S20,S23,S25,S29

Figures :

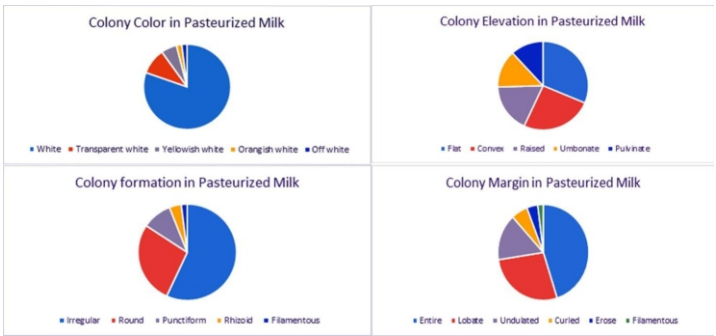


Fig : 1

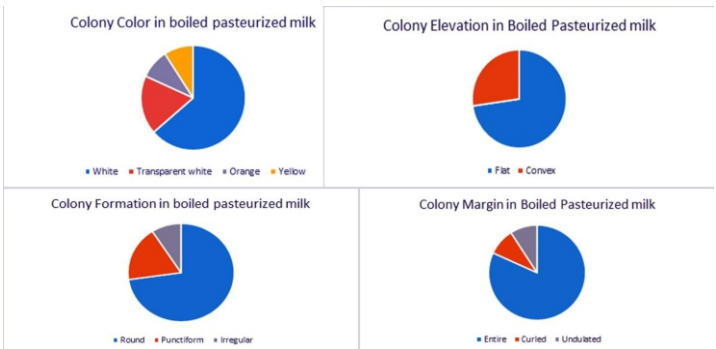
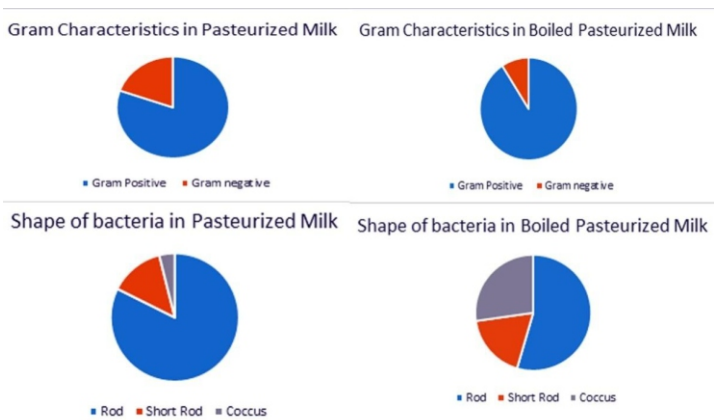


Fig : 2



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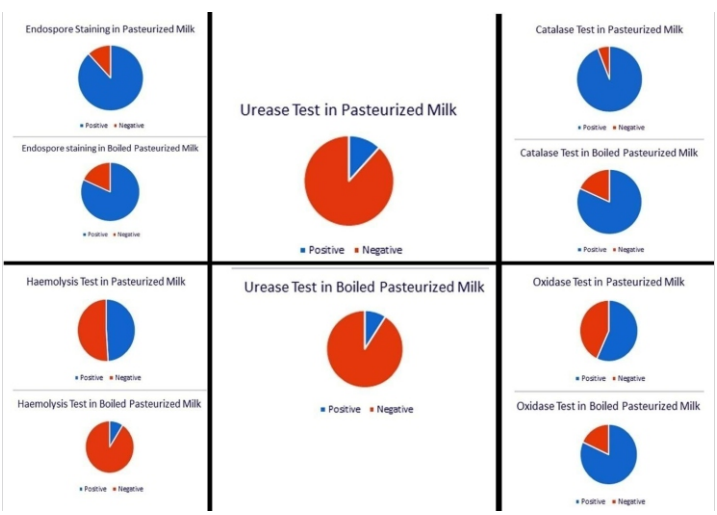

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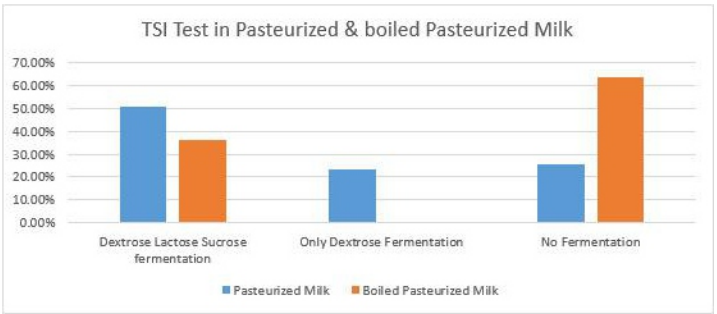

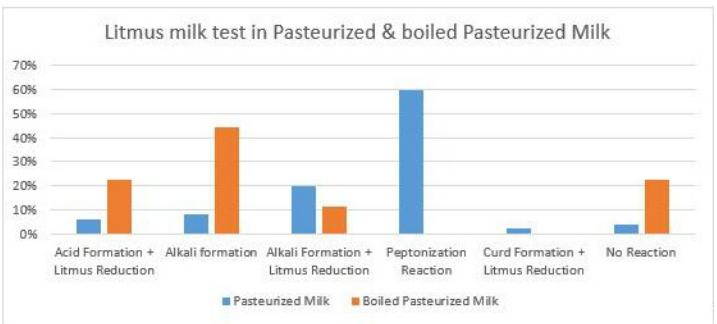

Fig: 5

Conclusions

The isolated pure cultures were screened by their colony colour, colony formation, colony elevation, and colony margin. The Gram characteristics and shape of these microorganisms also studied to ensure the screening process. Endospore staining of these microorganisms showed the thermostability of these organisms and the ability to form spores in adverse conditions.

The catalase test ensures the presence of hydrogen peroxidase enzyme in the microorganisms. So the microorganisms showed positive results might be from these genera- (*Staphylococci, Micrococci, Listeria, Corynebacteriumdiphtheriae, Burkholderiacepacia, Nocardia*, the family Enterobacteriaceae (*Citrobacter, E. coli, Enterobacter, Klebsiella, Shigella, Yersinia, Proteus, Salmonella, Serratia*), *Pseudomonas, Mycobacterium tuberculosis, Aspergillus, Cryptococcus, and Rhodococcus).*

The Oxidase test result confirms that the positive microorganisms are from Pseudomonas, Neisseria, Alcaligens, Aeromonas, Campylobacter, Vibrio, Brucella, Pasteurella, Moraxella, Helicobacter pylori, Legionella genera and the negative organisms are from Enterobacteriaceae genera.

This study presents a thorough microbiological study of boiled and un-boiled Pasteurized milk. Though it is a preliminary study, but ensures a positive contribution in the study of milk microbiology.

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