ISSN 2229-5518

Comparative Studies on the Prevalence of Bacterial Population and Physicochemical Analysis in Raw and Pasteurised Milk with special reference to Honey as a Natural Preservative

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Abstract: In this research, fifteen raw milk samples from different areas and ten pasteurized milk samples of various brands were collected and compared based on microbiological tests and physicochemical analysis. The milk samples were plated on nutrient agar medium and the isolates were then cultured on different media (EMB Agar, McConkey Agar, Skim Milk Agar). These cultured bacterias were then subjected to confirmative tests. The bacterias isolated were *Escheritia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus*. The milk samples were further subjected to physico-chemical analysis in which Fat content, Clearance Lactometric Reading, Solid Non Fat and Acidity were calculated. The quality of the milk samples were then analysed by Methylene Blue Reduction Test and Clot on Boiling Test.The isolated bacterias were also tested for their proteolytic and lipolytic property and were also subjected to catalase tset. Further the effect of honey as a natural preservative of milk was also analysed by studying the dilution of honey at which the maximum zone of inhibition was observed.

Index Terms : Raw milk, Pasteurised milk, Microbiological, Physicochemical analysis, Proteolytic, Lipolytic, Natural Preservative

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I INTRODUCTION

Milk is an important food with high nutritional value. Raw milk obtained from cow is pasteurised before packing in order to destroy the microorganisms present. Milk is an important growth medium for microorganisms when suitable temperature exists.It gets contaminated and spoiled very easily if handled carelessly and unhygienically The balanced diet milk becomes contaminated with several types of microorganisms which originate from the soil, water etc. Temperature plays a vital role in the spoilage of milk. Microorganisms such as psychotrophs may grow at a temperature of 7°C and they are distributed in diversified habitats such as soil, water, utensils and vegetation.

When the milk is stored under low temperature it gets contaminated and frequently undergoes spoilage due to proteinases and lipases released by the microbes present in the milk i.e psychrotropic bacteria. The presence of these organisms in milk indicate not only unsanitary conditions, but also the yard stick to measure the quality of the products. The psychrotrophs are readily killed by HTST

pasteurization but their extra cellular enzymes are heat stable to varying degrees when sufficient activity may remain to degrade the fats and proteins of milk.

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1.1 COMPOSITION OF MILK

Milk is a complex mixture of lipids, carbohydrates and proteins and many other organic compounds and inorganic salts dissolved in water.

Chemical composition of milk caries due to various factors such as species, breed of chemicals, climate etc

1.1 Water

The function of water in milk is to hold the solids of the milk partly in solution and partly in suspension.

1.1.2 Milk fat

Milk is a true emulsion of oil in water. Each globule of fat is surrounded by a thin layer which is composed of a complex lipid protein and a small amount of carbohydrate. The lipid portion includes both phospholipids and triglycerides. Milk fat is mixture of glycerides of fatty acids and other lipid materials present in milk are phospholipids.

1.1.3 Milk proteins

Milk contains casein and whey proteins. Casein occurs in milk as a colloidal protein- calcium phosphate complex. They are: α Casein: 66%, β Casein: 29%, γ Casein: 5%.

Whey proteins are made up of α-lactalbumin and β-lactoglobulin, serum albumin, enzymes and protease peptones. Whey also contains small amount of lactoferrin and serum transferrin.

1.1. Milk sugars:

The disaccharide lactose is the predominant and distinctive carbohydrate of milk but in addition there is low concentration of monosaccharide including glucose and galactose.

1.2. Salts

Chlorides, phosphates, citrates, sulphates and bicarbonates of sodium, potassium, calcium and magnesium are present.

1.3.Enzymes

a) **Alkaline phosphatase:** This enzyme is inactivated by normal pasteurisation procedures and its activity is tested to determine the effectiveness of pasteurisation.
b) **Lipase:** More than one type of lipase is present in milk. Milk lipids are responsible for rancid flavours in milk. Bacterial lipase is responsible for serious quality defects.
c) **Catalase:** It decomposes hydrogen peroxide to water and molecular oxygen.
d) **Casein hydrolysis:** Casein, the major milk protein, is a macromolecule composed of amino acid subunits linked together by peptide bonds (CO-NH). Before their assimilation into the cell, proteins must undergo step-by-step degradation into peptones, polypeptides, dipeptides, and ultimately into their building blocks as amino acids. This process is called peptonization or **proteolysis**, and it is mediated by extracellular enzymes called **proteases**. The function of these proteases is to cleave the peptide bond CO-NH by introducing water into the molecule. The reaction then liberates the amino acid.

2 MATERIALS AND METHODS

2.1 Sample Collection

Raw samples from different areas and pasteurised milk samples of different brands were collected during a period of January-March in the year 2012. The raw milk samples were collected from different areas of Chithamur (Kancheepuram District). The samples were collected whenever required and stored at refrigeration temperature for a period of 2-3 days if needed. The samples were collected in sterilised tubes under aseptic conditions.

.2.2 Serial Dilution: Milk samples were diluted by adding 9ml of saline in all 6 test tubes and 1ml of milk sample in first test tube then it subjected to serial dilution as 10^{-1},10^{-2},10^{-3}, 10^{-4},10^{-5} and 1 as control

2.3 Nutrient Agar Plate: Diluted milk samples were plated on nutrient agar and incubated at 37°C for 24 hours.

2.4 Isolation of Specific bacteria: Isolated bacteria were inoculated into appropriate selective media such as EMB agar, McConkey Agar and Skim Milk Agar.

2.5 Grams Staining : Gram staining was performed as per the standard procedure.

2.6 Motility Test :A drop of broth culture was placed in a cover slip. It was keep on a concave slide and observed under low power microscope. Corner of the cover slip was located. Hanging drop method was followed.

2.7 IMVIC TEST:

a) Indole Test: The indole test is performed by inoculating a bacterium into tryptophan broth, the indole produced during the reaction is detected by adding Kovac's reagent (dimethyl aminobenzaldehyde) which produces a cherry-red reagent layer.(Preparation of (1%) tryptone broth: Dissolved 10g of peptone in one litre of distilled water.Sterilized in the autoclave at 121°C for 15 minutes.)

b) Methyl Red Test: Inoculate the tubes of MRVP broth with the culture.Incubated the tubes along with control tubes at 37°C for 48 hours, following added 5 to 6 drops of methyl red solution into the test and control.Appearance of red colour was indicated, methyl red positive. .

c) Voges-Proskauer Test: Inoculate the test culture in the MRVP broth incubate the test and control tubes at 37°C for 48 hours.After 48 hours of incubation, add 1ml of 40% potassium hydroxide and 3ml of 5% alcoholic naphthol.Appearance of pink colour was considered as positive reaction of the test.

2.7 Citrate Test: Prepared simmon's citrate medium, dispersed in test tubes, sterilized at 121ºC for 15 minutes. Inoculate the cultures at 37ºC for 24 hours.

2.8 Catalase Test :1 ml of hydrogen peroxide was taken in 12 X 100 mm test tube. Small amount of bacterial culture was inoculated into the fluid with the help of a glass rod or plastic loop and the releases of air bubbles were observed and it was compared with the controls.

2.9 Proteolysis: Skim milk agar plates were prepared for inoculation. Bottom of the petridish was divided into two sections. Using sterile technique, single line streak inoculation of each test organism on the agar surface of its appropriately labeled section on agar plate was made. Plates were incubated in an inverted position for 24 – 48 hours at 37°C. Re incubate all negative cultures for an additional 5 days. Milk agar plate culture for the presence or absence of a clear area or zone of proteolysis, surrounding the growth of test organism was examined. Based on the observation, organisms capable of hydrolysing the milk protein casein can be determined.

2.10 Lipolysis: Tributyrin agar plates were prepared for inoculation. Bottom of the petridish was divided into two sections. Using sterile technique, single line streak inoculation of each test organism on the agar surface of its approximately labelled section agar plate was made and the other side was kept as control. Plates were incubated in an inverted position for 24-48 hours at 37°C. A clear zone surrounding the area of the inoculated organism shows positive result for lipase test thus indicating the lipase producing property of the organism.

2.11 Phosphatase Test: The test is used to judge the efficiency of pasteurization of milk. According to the accepted practise in the country, milk for liquid trade is pasteurized by the holding method (63 to 66°C) for 30 minutes or by the high temperature short time (72.2 to 72.8°C) for 15 seconds. To test whether the heat treatment by either of these methods was properly carried out, the treated milk is subjected to the phosphatase test, which helps to indicate the presence or absence of phosphatase enzyme.

2.12 Methylene Blue Reduction Test

Three clean and sterilized test tubes were taken. To each of the test tubes 10 ml of milk to be tested was added. Second and third tubes were placed in boiling waterbath for 3 minutes to destroy the natural reducing system of the milk. These two tubes will serve as controls. Then 1 ml of certified methylene blue of 1: 25,000 dilutions to the first tube and 1 ml of tap water to the third tube was added. The milk in the first two tubes will look blue and in the third tube, the milk will remain white. Incubate all three tubes in waterbath at 37° C and note the time. The second and the third tubes which serve as controls will not show any colour change. Milk in the second tube will remain blue and milk in the third tube will remain white, while milk in the first tube will gradually become colourless except at the top where the milk is in contact with air. The second tube as control will indicate when colour change starts in the first tube and the third tube will indicate when the colour change is complete.

Table I: Reference table for MBRT

S. No	Reduction time	Quality
1.	More than 8 hours	Excellent
2.	6 – 8 hours	Good
3.	2 – 6 hours	Fair
4.	Less than 2 hours	Poor

2.13 Clot on Boiling Test: Boil a small amount of milk in a test tube or other suitable container. If there is clotting, coagulation or precipitation, the milk has failed the test. This test is for too acid or abnormal milk. If a milk sample fails in the test, the milk must contain many acid or rennet producing microorganisms or the milk has an abnormal high percentage of protein like cholesterol milk. Such milk cannot stand the heat treatment in milk processing and must be rejected.

2.14 Acidity Test: The natural acidity of milk is 0.16-0.18%. Figures higher than this signifies developed acidity due to the action of bacteria on milk sugar. 9ml of the milk measured into the conical flask, 1ml Phenopthalein is added and then slowly from the burette, 0.1N Sodium hydroxide is added drop wise under continuous mixing, until a faint pink colour appears. The number of millilitres of Sodium hydroxide solution divided by ten expresses the percentage of lactic acid.

2.15 Lactometer Test:. Mix the milk sample gently and pour it into a measuring cylinder. Let the lactometer sink slowly into the milk. Read and record the last lactometer degree(°L) just above the surface of the milk is different from the calibration temperature (calibration temperature may be 20°C) of the lactometer, calculate the temperature correction. For each degree °C above the calibration temperature add 0.2°L; for each °C below calibration temperature subtract 0.2°L from the record lactometer reading.

2.16 Fat Test: Fat content in the milk samples were tested with the help of Fatometer. The fatometer uses a solution which is made up by mixing 100ml of Fatometric solution in 10 litre of water.

The fat content was also determined by centrifugation method. Here 10ml of 90% Sulphuric Acid, 10.75ml of milk and 1ml of Amyl Alcohol were taken in a jargon tube and mixed well. This was then subjected to centrifugation at 1200rpm for 5minutes. From that the fat level can be measured.

2.17 Solid Non Fat Test: It can be calculated from the CLR value, ie.. SNF=CLR/4

2.18 Total Fat: The total fat content in the milk sample is the sum of fat content and SNF content in the milk sample.

2.19.Anti Microbial Activity of Honey

It was found that artificial honey had no inhibitory effect on the growth of either catalase positive or negative bacteria In this work, artificial honey was diluted with sterilized water at a rate of 5^{-1} to 5^{-4}. Bacterial isolates from milk sample were streaked on nutrient agar plates. The anti-microbial activity was determined using the agar well diffusion assay method. Using sterile micropipette, 20µl of the diluted artificial honey sample was poured onto each well along with sterilized water as control into a well. The effect of honey was tested against different bacterial isolates from the milk sample. The plates were incubated at 37°C for 24hours. After incubation the plates were examined for evidence of zone of inhibition, which appears as a clear area around the wells. The diameter of such zones of inhibition was measured using a meter ruler.

3. RESULTS

TABLE – 2 METHYLENE BLUE REDUCTION TEST FOR RAW MILK SAMPLES

Sample No.	Reduction time (hours)	Grade
1	2	Fair
2	30 Minutes	Poor
3	1	Poor
4	1½	Poor
5	1	Poor
6	2½	Fair
7	2	Fair
8	1	Poor
9	2	Fair
10	1½	Poor
11	45minutes	Poor
12	1	Poor
13	3½	Fair
14	1	Poor
15	1	Poor

TABLE – 3 METHYLENE BLUE REDUCTION TEST FOR PASTEURISED MILK SAMPLE

Sample No.	Reduction time (hours)	Grade
1	4	Fair
2	5	Fair
3	4	Fair
4	3½	Fair
5	6	Good
6	4	Fair
7	3	Fair
8	4½	Fair
9	6½	Good
10	5	Fair

TABLE – 4 PHYSICOCHEMICAL ANALYSIS RESULT FOR RAW MILK SAMPLES

Sample	Acidity	Fat	CLR	SNF
1	0.142	4.6	26.5	6.625
2	0.145	3.9	25.5	6.37
3	0.150	4.6	27	6.75
4	0.158	4	28	7
5	0.143	3.5	20	5
6	0.144	4.2	27.5	6.875
7	0.149	4	26	6.500
8	0.152	3.8	28.5	7.125
9	0.160	4.3	26.5	6.625
10	0.156	4	25	6.25
11	0.161	3.5	21.5	5.375
12	0.149	4.2	27.5	6.875
13	0.158	4	28	7
14	0.161	3.5	21.5	5.375
15	0.146	3.9	25.5	6.37

TABLE – 5 PHYSICOCHEMICAL ANALYSIS RESULT FOR PASTEURISED MILK SAMPLES

Sample	Acidity	Fat	CLR	SNF
1	0.144	4.7	30	7.5
2	0.162	3.1	32	8
3	0.144	4.7	30	7.5
4	0.081	3.4	33	8.25

5	0.135	3.8	33	8.25
6	0.146	4.2	28	7
7	0.158	4.2	30	7.5
8	0.161	3.9	31	7.75
9	0.146	4.3	29	7.25
10	0.155	4.1	26	6.5

Acidity : 0.144-0.166

CLR : 20-40

The Clot on Boiling test showed negative result for all samples.

TABLE – 10 Bacterial isolates in Milk

	Indole	**Methyl Red**	**Voges-Proskauer**	**Citrate**
Escherechia coli	+	+	-	-
Staphylococcus aureus	-	+	+	-
Bacillus cereus	+	-	-	+
Pseudomonas aeruginosa	-	-	+	-

GRAPH 1-MBRT – Raw milk

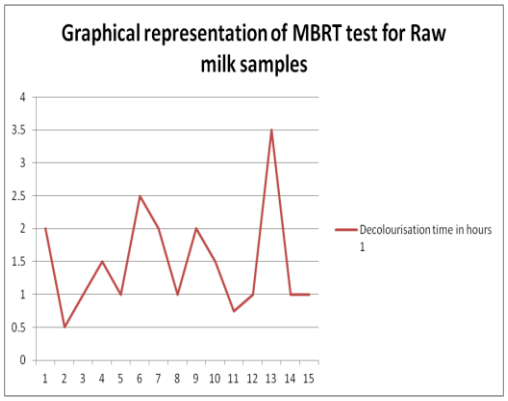


TABLE – 6 PHOSPHATASE TEST RESULT

SAMPLE No	**PHOSPHATASE TEST**
1	Negative
2	Negative
3	Negative
4	Negative
5	Negative
6	Negative
7	Negative
8	Negative
9	Negative
10	Negative

GRAPH 2-MBRT –PASTEURIZED MILK

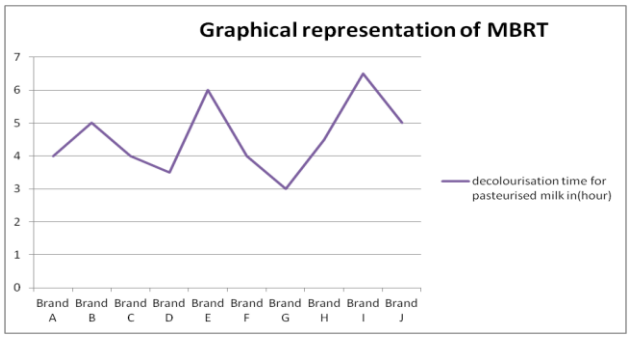
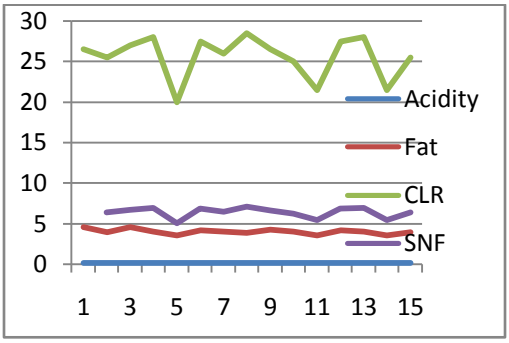


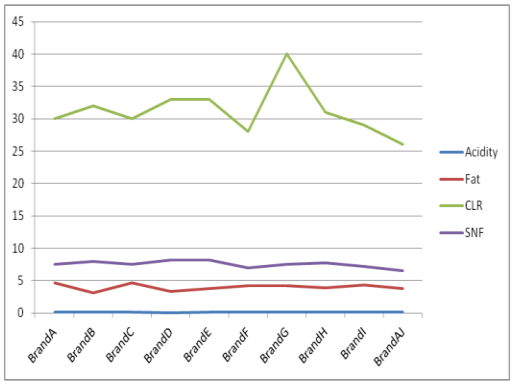
TABLE- 7

MICROBIOLOGICAL TESTS RESULT

GRAPH 3- PHYSICOCHEMICAL ANALYSIS RESULT FOR RAW MILK



GRAPH 4- PHYSICOCHEMICAL ANALYSIS RESULT FOR PASTEURISED MILK SAMPLES



4.DISCUSSION

The MBRT results are tabulated in Table2&3 for raw and pasteurised milk samples respesctively. In raw milk samples, five samples were fair and ten samples were poor. In pasteurised milk samples, two samples were good and remaining eight were fair enough. Chatterjee et.al (2006) reported excellent quality milk both in raw and pasteurised samples. There are no chances of excellent quality milk in case of raw samples because of the environmental conditions and the cattle feed. In this work, no such excellent quality milk was determined.

With reference to physicochemical analysis, Table-4&5 shows the result for raw and pasteurised milk samples respesctively. In raw milk, the acidity is usually between 0.144-0.166 , CLR is between 20-30. The pasteurised samples show almost the same acidity values, their CLR values lie between 25-40. There is a variation in SNF value for raw and pasteurised milk samples. The pasteurised samples show greater SNF value.

Further, the pasteurised milk samples were subjected to Phosphatase test in order to test the efficiency of pasteurisation. This showed that the pasteurisation was upto the mark but still there was prevalance of microorganism which may be due to the improper handling and unhygenic conditions.

With reference to microbiological tests done on raw and pasteurised milk samples, the results depicted in Table-III shows the different percentage of bacteria with different characterestics. Four different bacteria were isolated from the samples collected.

Table IV shows the results for the confirmatory test result done to confirm the bacteia. The IMVIC test was done to check the biochemical properties of the isolates.

Further , the antimicrobial activity of artificial honey against the isolates from milk samples showed positive result. N S A Krushna et.al (2005) reported that artificial honey had no inhibitory effect on the microorganisms isolated from the milk samples. In this work, artificial honey depicted a zone of inhibition for *Bacillus cereus* isolaed from milk which proved that it can be used as a natural preservative for milk instead of chemicals like formaldehyde.

5.CONCLUSION

From the results obtained, the following conclusions can be made: Pasteurisation of milk does not alter its physicochemical properties to a great extent with reference to acidity and fat content.The pasteurisation is effective in killing the microbes present in the raw milk samples to a great extent but not completely. The milk processing companies should concentrate on hygeinic conditions during the process. The peservation of milk should be given more attention. They should be preserved natirally instead of chemical preservation which affects the public health. The artificial honey showed a good resistance against the isolate from the milk sample which makes it very effective as a natural preservative.

6.ACKNOWLEDGEMENTS

We are thankful to our Mrs.Meenakshi Annamalai, Director,Karpaga Vinayaga College of Engineering and technology for providing research facility for the successful completion of the project work. The authors would like to acknowledge Prof.T.rangarajulu,Dean, KVCET and V.C.Ravichandiran,Advisor,,KVCET for their Valuable guidance.

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