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An Applied Research Method on Assembling Milk Spoilage Bacteria from Different Milk Samples

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Abstract Worldwide, Pasteurized Milk and Milk products are popular food to consumers. A lot of works have been concerned out on Pasteurized and UHT milk during the last few years in different concerns of the world. There are a lot of information in the world related to characters of the different microorganisms involved, although Pasteurized milk is one of the most favorite and liking food item to the young generation in the country. Therefore Pasteurized milk and milk products storage for long time should always be avoided. As most of the bacteria are able to produce toxins, it is necessary to monitor strictly Pasteurized milk and milk products and then certify them for human consumption. It is the author's (Chowdhury AP *et.al.*) description that pasteurization is simply a quick fix that allows large cartels to profit from the sales of milk. So also for its demand in the market many companies and renown dairy farms have been prepared it without proper maintaining its nutritional quality, hygienic condition. To analyze the hygienic condition in Pasteurized milk, research works prepared by well known and established Research laboratories in the Chittagong, Bangladesh.

Keywords: *alkaline phosphatase, somatic cell count, coli form count, microscopic factor*

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1. Objectives

Methodological procedure is applied to identify of the selected bacterial isolates on the basis of their cultural, morphological and physiological characteristics.

2. Introduction

Scientist developed their technology which is beneficial to human being for hygienic consumption of milk and milk products. In the light of these subjects related literature has been reviewed. "Lorenzen and Martin [1] studied evaluation of alkaline phosphatase detection in dairy products using a modified rapid chemiluminescent method and official methods". "Desbourdes and Nicolas [2] discussed about Phosphatase activity in cheese for Milk and Milk Products". "Wilinska and Bryjak [3] augmented in International Dairy Journal that Alkaline Phosphatase in cow and non cow milk and cheese determination of enzyme activity as an indicator for the completeness of the pasteurization process". "Fox and Kelly [4] showed in International Dairy Journal that the use of alternative analytical methods is acceptable when the alkaline phosphatase determination methods are validated against the reference method in accordance with internationally accepted protocols". "Vamvakaki and Zoidou [5] represented about Small Ruminant Research, that Determination of alkaline phosphatase activity". Analytical report based on Milk and milk products where as Mc Martin *et al.* [6] proved important pathogens including *Listeria monocytogenes*, *E. coli*, *Salmonella enteritidis*in pasteurized milk. Godden *et al.* [7] showed a recent field trial that when colostrums, heat-treated at 140°F for 60 minutes, was fed to calves, these calves experienced significantly improved efficiency of absorption of colostrums antibodies and had significantly higher serum IgG concentrations at 24 hours after birth, as compared to calves fed raw colostrums. Hagman *et al.* [8] studied about this benefit is thought to be due to the fact that there were significantly fewer bacteria present in the heat treated colostrums to interfere with antibody absorption across the gut. Stable *et al.* [9] was taken the lead in researching UHT milk and discussed how UHT processing and subsequent storage causes several changes affecting the shelf life of UHT milk. The changes include whey protein denaturation, protein interaction, lactose protein interaction, isomerisation of lactose. Green and Godden *et al.*

[10] determined in most situations, heat treating colostrums at 140°F (60°C) for 60 minutes in a commercial batch pasteurizer should be sufficient to maintain IgG concentrations while eliminating. Poulsen *et al.* [11] denoted that bacterial contamination of pasteurized milk is a concern because pathogenic bacteria can act directly to cause diseases such as septicemia. "Ardo and Lindblad

[12] proved that it is possible to discriminate native ALP, which is less heat stable from ALP produced by microorganisms by testing cheese after repasteurization at 62.8°C by 30 minutes". The positive result is caused by micro flora. "Steele and Walz [13] showed that Pasteurized milk can also represent one of the earliest potential exposures of dairy calves to infectious agents, including *Mycoplasma sp*., *Mycobacterium paratuberculosis*, fecal coliforms and *Salmonella sp."* Los Angeles County Board of Supervisors concerning outbreaks from pasteurized milk in 1997, 28 persons ill from *Salmonella sp.* in California, and 1996, 46 persons ill from *Campylobacter sp.* and *Salmonella sp.* in California. Then 1994, 105 persons ill from *E. coli* and *Listeria sp.* in California March of 1985, 660 confirmed cases of *Salmonella typhimurium* illness. Over 200,000 people ill from *Salmonella typhimurium*. In 1985, 142 cases and 47 deaths traced to pasteurized Mexican style cheese contaminated with *Listeria monocytogenes*. Then in 1985,1500 persons ill from *Salmonella sp.* infection. August of 1984, approximately 200 persons became ill with a *Salmonellatyphimurium* from consuming pasteurized milk. In 1983, over 49 persons with *Listeria sp.* Illness have been associated with the consumption of pasteurized milk in Massachusetts in 1983, 28 persons ill from *Salmonella sp.* Infection. And 1982,172 persons ill (100 hospitalized) from a three Southern state area from pasteurized milk, over 17,000 persons became ill with *Yersinia enterocolitica* from pasteurized milk bottled in Memphis, Tennessee.

3. Materials and Methods

3.1. Samples Collection

In the present study two types of milk samples were collected - Raw milk samples were collected from 40 (forty) healthy cows of Senowara Dairy farm, Chittagong, and UHT samples - Farm Fresh, Milk Vita, Aarong and Pran were collected from four suppliers.

3.2. Screening of Raw MilkSamples for Mastitis and Somatic Cells

Raw Milk samples collected fromSenowara Dairy Farm were used to determine whether they were having Mastitis and somatic cells. For this purpose, milk samples were studied details. The California Mastitis Test (CMT) is a rapid, accurate, cow side test that helps to determine somatic cell counts (SCC) of raw milk from a specific cow. Los Angeles County Board of Supervisors has developed the CMT.

Somatic Cell Count (SCC) is used as an indicator of the quality of raw milk (i.e., its suitability to make high-quality milk products). Somatic cells are primarily white blood cells (i.e., leukocytes). The number of somatic cells may increase as a result of udder infection (e.g., mastitis) or teat/udder injury and varies due to many factors, including the cow's age, lactation stage, season and stress.

3.2.1. Samples Preparation

Raw milk of healthy cows from Senowara Dairy Farm was denoted as Sample RM; half of the raw milk samples were pasteurized in a home pasteurizer and denoted them as HPM. Commercially pasteurized (UHT) milks - Farm Fresh, Milk Vita, Aarong and Pran were denoted as UHT respectively. In this method verified results were studied then mastitis negative samples denoted for rejection. Following the tested mastitis samples RM (Selected No.26) should to take for Pasteurization by dint of Home Pasteurizer machine. Recommended samples should to take for Pasteurization process due to presence of Mastitis causing Microorganisms of milk.

(i) Phosphatase Test by Optimized Standard Method

In the presence of magnesium and zinc ions p-Nitro phenyl phosphate is hydrolyzed by Phosphatase to form phosphate and p-Nitro phenol. The p-Nitro phenol released is proportional to the Alkaline Phosphatase activity and can be measured photometrical to determine the alkaline phosphates level presence in the milk. Colorimetric assay in accordance with a standard method followed by samples and then addition of Buffer solution (R1) and addition of buffer/substrate (R2).

(ii) Coliforms Test

The sample bottle is shaken 25 times in up & down movement of about a foot in a time interval not exceeding 7 seconds.

1:100, (1ml milk + 99ml dilution blank)
1:1000, (1 ml + 99 ml dilution blank)
1:10000, (1ml +99 ml dilution blank)

And so on dilution were made in the sterilized buffered distilled water according to the following dilution scheme. Before transferring from a dilution bottle into Petri dishes or other dilution bottle, the bottles were shakes as described for the original milk sample. The pipette was allowed for few seconds to drain them gently and last drop was blow out and touch the tip of the pipette to a dry spot on the glass, to remove the remaining materials while increasing with dilutions into Petri dishes or into the other dilution bottle. As soon as the dilutions are in the Petri dishes, the mouth of agar bottle is flamed & poured approximately 10-15 ml of agar in each dishes (1/8 inch depth). The agar is mixed with dilution gently by rotating & tilting method.

A secondary layer is made with the same agar by placing 4-5 ml in each of the Petri dishes & allow sometimes for solidification. To prevent surface growth & spreading of colonies, allow sometimes for solidification, bacteria lie between 2 layers, after solidification, the Petri dishes are inverted & placed them in the incubator at 32°C for 24hrs.

After 24 hrs a plate is selected having a sorted colony and count them with measure from 0.5 mm in diameter.

(iii) Direct Microscopic Count (Breed Count Method)

Direct Microscopic Count (Breed Count Method) gives us the estimate the no. of viable bacteria present. Standard method has been established using a convenient temperature & a simple culture medium for the detection of the no. of bacteria that grow under the condition specified. To determine the area of field the sq. radius to be multiplied by 3.1416 that is the area in sq. mm. Convert the area of 1 field from sq mm. to sq.cm. Determine the no. of field in 1 sq.cm, is to be divided by the area of 1 field. Since 0.01 ml of milk was spread over 1 cm sq. area, the no. of fields to be multiplied by 100 to determine the no. of microscopic field per ml of milk.

(iv) Standard Plate Count

Standard Plate Count gives us the estimate the no. of viable bacteria present. Standard method has been established using a convenient temperature & a simple culture medium for the detection of the no. of bacteria that grow under the condition specified. To detect the no. of bacteria present per ml or gm of milk/ milk sample to detect the quality of milk.

(v) Isolation and Purification of the Microorganisms

a) Preparation of the dilution

The microbial colonies were isolated by serial dilution plate procedure (Foster et al. 1958). For this purpose, 5gm of the chicken sandwich was taken in 90ml of the sterile distilled water in a sterile conical flask. It is used as the 10^{-1} dilution. Further dilutions were prepared up to 10^{-6} from the 10^{-1} dilution in sterile distilled water.

b) Media

For the isolation and enumeration of microorganisms particularly bacteria, nutrient agar media was used although the study.

c) Plating

Pour plating in duplicate plates were made for each diluted sample in the following. One ml of each of the dilution was added to about 20 ml of sterile melted agar (40°C) medium in sterilized Petri plates and mixed thoroughly by rotating the plates both clockwise and anticlockwise. In this way, all plating's were carried out from all serial diluted samples.

d) Incubation

After solidification of the medium, the plated were incubated (Binder Incubator) at inverted position at 37±1°C for 24 to 72 hours.

(vi) Enumeration of Bacteria

After 24-48 hours of incubation, the plates having 30 to 300 colonies were selected for counting. The selected plates were placed on a colony counter (Stuart Scientific, UK) for counting or counting is normally done by visual observation. The colonies or viable aerobic bacterial count per ml or gm were calculated by multiplying the average number of colonies per plate by the reciprocal of the dilution (Collins and Lyne 1984). The calculated results would be as colony forming units (cfu) per ml or gm of sample.

(vii) Isolation of the Discrete Bacterial Colonies

Isolation of the discrete bacterial colonies was carried out immediate after counting of the colonies. On the basis of their morphology, several different colonies were selected for the purpose. The selected colonies were marked and their characteristics were studied depending on the various points' viz. form, elevation, margin, surface, color etc. (Eklund and Lankford 1967; Bryan 1950). Then the marked and observed bacterial colonies were transferred to nutrient agar slant aseptically for purification.

(viii) Purification of the Isolates

The isolated organisms were purified through repeated plating method. Both pour plate and streak plate methods were used for the purpose. Media used for this purpose were nutrient agar (NA). When a plate yielded only one type of colony the organism were considered to be pure. The purification of the isolates was also confirmed under microscopic observation. The pure bacterial isolates were preserved on the nutrient agar slant aseptically for further studies.

(ix) Maintenance and Preservation of the Isolates

The purified bacterial isolates were maintained on nutrient agar slants during the course of investigation. The culture tubes (slant tube) were kept in polythene bags. The bags were tied up and preserved in a refrigerator at 4°C as stock culture. These isolates were transferred to fresh medium periodically.

3.2.2. Final Selection of the Isolates

Final selection was made on the basis of their colonial morphology on the agar plate, on agar slants and their microscopic features under the microscope.

3.2.3. Morphological and Cultural Studies of Selected Isolates

Identification of the microorganisms was a sequential process, which included a series of different types of experiments. In the present study the following sequence of experiments were carried out for the presumptive identification of the isolates.

3.2.4. Biochemical Studies of the Selected Isolates

The physiological characters of all the isolates i.e. their growth at different temperature, pH, their salt (NaCl) tolerance and heat resistance were studied by Sneath *et al.* [14] in nutrient agar slants containing different concentration of sodium chloride (0, 1, 2, 3, 4, 5, 6, 7, 8 and 10) were inoculated and incubated at 37°C for 48 to 72 hours. The growth of organisms at different concentration of NaCl was then compared with the control. Casein is a milk protein. Many organisms have the capacity to hydrolyze casein. For this test, 1 ml of sterile skim milk was taken in a sterilized Petri plate and then melted agar was poured into the Petri plate, mixed thoroughly and allowed to solidify. After solidification, the plates were inoculated by streaking and incubated at 37°C for 2 to 4 days. A clear transparent zone around the colonies indicated the production of caseinase enzyme by the bacteria, described by Collons and Lyne [15] that could hydrolyze casein. The enzyme Catalase is capable of decomposing hydrogen peroxide into water and molecular oxygen described by Claus [18].

$$2H_2O_2 = 2H_2O + 2O_2.$$

For this test, nutrient broth tubes were inoculated with 48 to 72 hours old culture and incubated at 37°C for 24 to 48 hours. After incubation a few drops of hydrogen peroxide was added to each of the tubes. Production of bubbles indicated the positive result. Hydrogen sulphide reacts with lead acetate to form lead sulphide (black in color). This property of hydrogen sulphide had been utilized in the demonstration of its production in the peptone iron agar medium (ISP-VI) by microbes reffered to Tresner and Danga [16]. Blackening of the lead acetate paper indicated the production of hydrogen sulphide. Microorganisms having the capability of producing the enzyme amylase can hydrolyze starch to maltose (SAB 1957). By this test the presence of amylase in the organism can be detected. Development of deep blue color indicated that starch had not been hydrolyzed. Gelatin is a weak proteinaceous colloidal substance studied by Collons and Lyne [15].The liquefaction of the inoculated tubes indicated the presence of the enzyme gelatinase.

This test detects the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or some other nitrogenous compound, such as molecular nitrogen (N_2), using the enzyme nitrate reductase. Nitrate (NO_3) may be reduced to several different compounds, either by anaerobic respiration or by denitrification. This test is used to detect whether or not the reduction has taken place.

4. Results

Table 1. Standard of Coli form Bacteria (Los Angeles County Board of Supervisors)

Samples	Grades	Standard
Raw milk	Certified	Less than100/ml
Home Pasteurized milk	Certified	less than 10/ml
UHT milk	Certified	≤ 1/ml

Table 2. Direct Microscopic Count (Los Angeles County Board of Supervisors)

Samples	Grades	Standard
Raw milk	Less than 2 million	satisfactory
Home pasteurized milk	Less than 0.5 million /ml	Accepted
UHT	Less than 0.5 million /ml	Accepted

Calculation:
Average no. bacteria= Y.
No. of bacteria =MF × Y (MF= Microscopic Factor)
Calculation of Microscopic Factor:
FDR=18
=18× 0.01 mm (each division =0.01)
= 0.18mm
Radius r,= 0.18/2=0.09mm
Area of 1 field= πr^2
=3.1416 × $(0.09)^2$ sq.mm.
=3.1416 × 0.0081 sq mm.
= (3.1416 × 0.0081)/100 sq cm.
(3.1416 × 0.0081)/100 sq cm.
cover the area of 1 field. Therefore,
1 sq cover the $1/(3.1416 \times 0.0081)/100$
$= 100/3.1416 \times 0.0081$
0.01ml of milk distributed in
$= (3.1416 \times 0.0081)/100$ field
Therefore 1 ml milk distributed in= 100/ (3.1416 × 0.0081× 0.01)
=392794.2491
microscopic factor for 1 ml milk
.MF = 392794.2491
No. of bacteria= 392794.2491 × Y

Table 3. Results of Standard Plate Count(Los Angeles County Board of Supervisors).

Samples	Grades	Standard
Raw milk	Less than 2 million	satisfactory
Home pasteurized milk	Less than 0.5 million /ml	Accepted
UHT	Less than 0.5 million /ml	Accepted

Table 4. Enumeration of bacterial colonies by different counting methods

No. of Samples	Coli form Count Cfu. /ml (million)	Direct Microscopic Count Cfu. /ml (million)	Standard Plate Count Cfu. /ml (million)	Grades	Comments
RM1	17	2.18	1.34	Certified	Accepted
RM2	26	1.10	1.16	Certified	Accepted
RM3	15	1.05	1.00	Certified	Accepted
RM4	45	1.34	0.34	Certified	Accepted
RM6	55	1.60	0.40	Certified	Accepted
RM7	32	1.79	1.65	Certified	Accepted
RM9	37	1.58	1.50	Certified	Accepted
RM12	45	1.34	1.28	Certified	Accepted
RM16	65	1.84	1.00	Certified	Accepted
RM17	24	1.27	0.17	Certified	Accepted
RM18	73	1.65	1.00	Certified	Accepted
RM20	37	1.17	1.12	Certified	Accepted
RM21	82	1.04	1.00	Certified	Accepted
RM22	12	2.18	1.55	Non certified	Increase No. ofColonies(DMC)
RM23	44	1.85	0.25	Certified	Accepted
RM25	36	1.08	1.00	Certified	Accepted
RM26	28	2.72	1.72	Non certified	Increase No. ofColonies(DMC)
RM27	69	1.66	1.00	Certified	Accepted
RM29	45	1.94	1.28	Certified	Accepted
RM31	76	1.28	1.07	Certified	Accepted
RM32	66	1.74	1.02	Certified	Accepted
RM34	56	1.48	1.00	Certified	Accepted
RM35	23	1.00	1.05	Certified	Accepted
RM37	18	1.19	1.34	Certified	Accepted
RM38	34	1.55	1.09	Certified	Accepted
RM40	62	1.71	1.28	Certified	Accepted

No. of Samples	Coli form Count Cfu. /ml (million)	Direct Microscopic Count Cfu. /ml (million)	Standard Plate Count Cfu. /ml (million)	Grades	Comments
HPM1	8	0.14	0.11	Certified	Excellent
HPM2	6	0.20	0.08	Certified	Excellent
HPM3	1	0.05	0.34	Certified	Excellent
HPM4	5	0.28	0.81	Non certified	Increase No. ofColonies(SPC)
HPM6	7	0.30	0.35	Certified	Excellent
HPM7	9	0.22	0.08	Certified	Excellent
HPM9	8	0.38	0.45	Certified	Excellent
HPM12	5	0.16	0.11	Certified	Excellent
HPM16	8	0.25	0.20	Certified	Excellent
HPM17	2	0.27	0.26	Certified	Excellent
HPM18	7	0.31	0.15	Certified	Excellent
HPM20	5	0.12	0.10	Certified	Excellent
HPM21	9	0.35	0.03	Certified	Excellent
HPM22	1	0.20	0.55	Non certified	Increase No. ofColonies(SPC)
HPM23	4	0.13	0.18	Certified	Excellent
HPM25	5	0.47	0.38	Certified	Excellent
HPM26	8	0.33	0.22	Certified	Excellent
HPM27	6	0.26	0.30	Certified	Excellent
HPM29	5	0.37	0.18	Certified	Excellent
HPM31	7	0.20	0.15	Certified	Excellent
HPM32	3	0.42	0.20	Certified	Excellent
HPM34	8	0.12	0.10	Certified	Excellent
HPM35	2	0.29	0.11	Certified	Excellent
HPM37	8	0.10	0.36	Certified	Excellent
HPM38	4	0.25	0.70	Non certified	Increase No. ofColonies(SPC)
HPM40	9	0.43	0.35	Certified	Excellent
UHT1	1	0.03	0.01	Certified	Satisfactory
UHT2	0	0.07	0.05	Certified	Satisfactory
UHT3	0	0.02	0.01	Certified	Satisfactory
UHT4	0	0.14	0.10	Certified	Satisfactory
UHT5	2	0.20	0.08	Non certified	Increase No, ofColi form count

Raw Milk

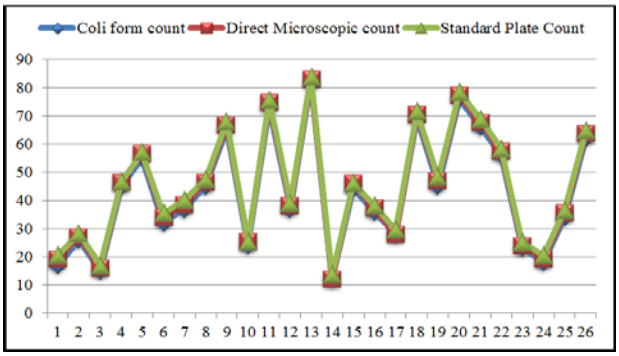


Figure 1. Graphical presentation showed highest range of discreet colonies in raw milk (RM) samples.

Home Pasteurized Milk

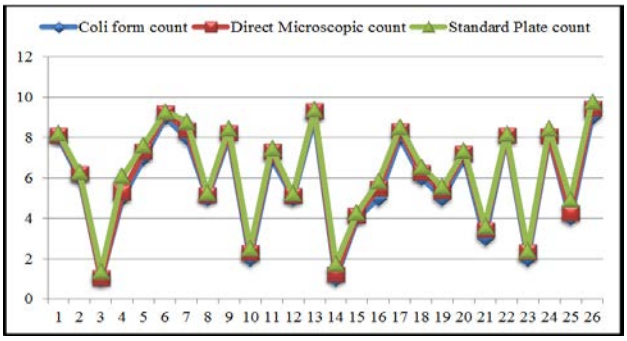


Figure 2. Assembling data represented accepted range of discreet colonies in home pasteurized milk (HPM) samples.

UHT Milk:

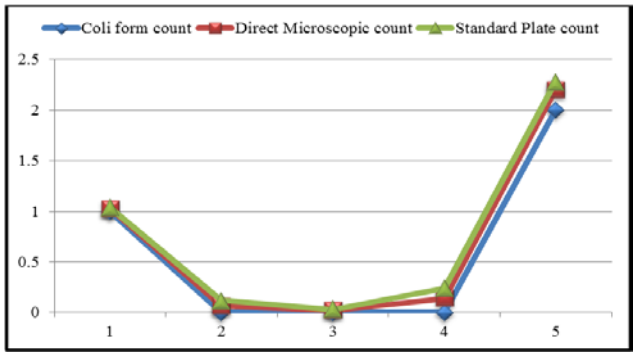


Figure 3. Assembling data represented fluctuating range of discreet colonies in ultra heat treated (UHT) milk samples.

Pie-diagram:

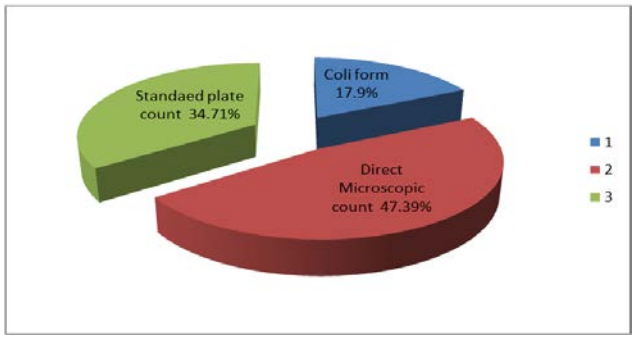
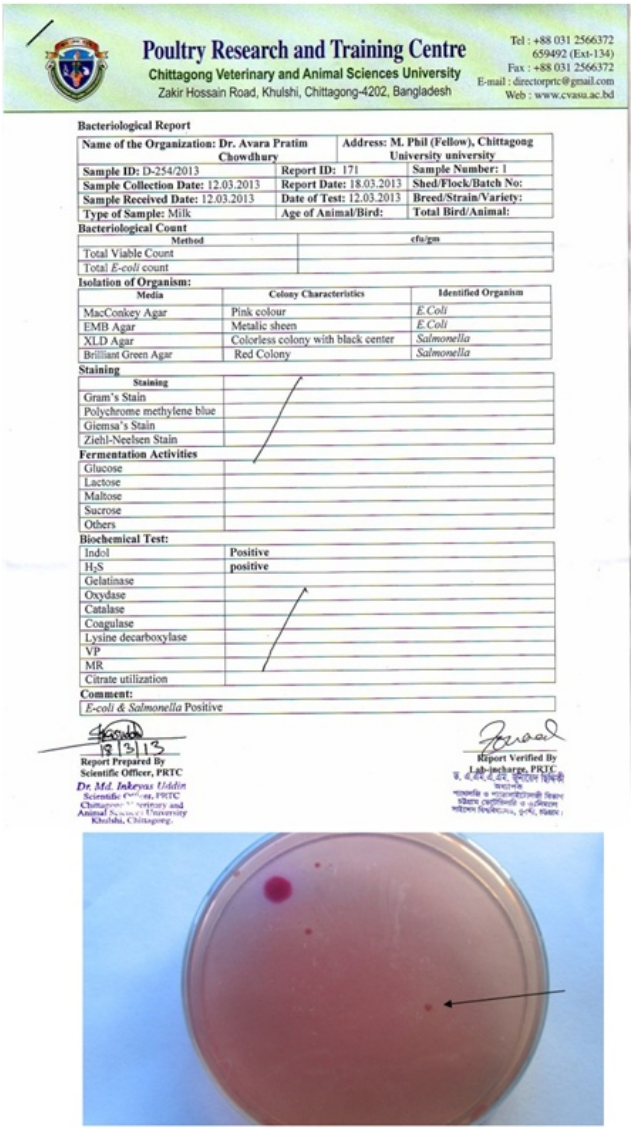


Figure 4. Total means percentages on data were represented for contaminated or re contaminated bacterial colonies from different milk samples



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Bacteriological Report

Name of the Organization: Dr. Avara Pratim Chowdhury		**Address: M. Phil (Fellow), Chittagong University university**
Sample ID: D-254/2013	**Report ID:** 171	**Sample Number:** 1
Sample Collection Date: 12.03.2013	**Report Date:** 18.03.2013	**Shed/Flock/Batch No:**
Sample Received Date: 12.03.2013	**Date of Test:** 12.03.2013	**Breed/Strain/Variety:**
Type of Sample: Milk	**Age of Animal/Bird:**	**Total Bird/Animal:**

Bacteriological Count

Method	cfu/gm
Total Viable Count	
Total *E-coli* count	

Isolation of Organism:

Media	Colony Characteristics	Identified Organism
MacConkey Agar	Pink colour	*E.Coli*
EMB Agar	Metalic sheen	*E.Coli*
XLD Agar	Colorless colony with black center	*Salmonella*
Brilliant Green Agar	Red Colony	*Salmonella*

Staining

Staining	
Gram's Stain	
Polychrome methylene blue	
Giemsa's Stain	
Ziehl-Neelsen Stain	

Fermentation Activities

Glucose	
Lactose	
Maltose	
Sucrose	
Others	

Biochemical Test:

Indol	Positive
H_2S	positive
Gelatinase	
Oxydase	
Catalase	
Coagulase	
Lysine decarboxylase	
VP	
MR	
Citrate utilization	

Comment:
E-coli & Salmonella Positive

Report Prepared By
Scientific Officer, PRTC
Dr. Md. Imkeyas Uddin

Report Verified By
Lab-incharge, PRTC

Figure 5. E.coli colonies in MacConkey agar (sample-3)

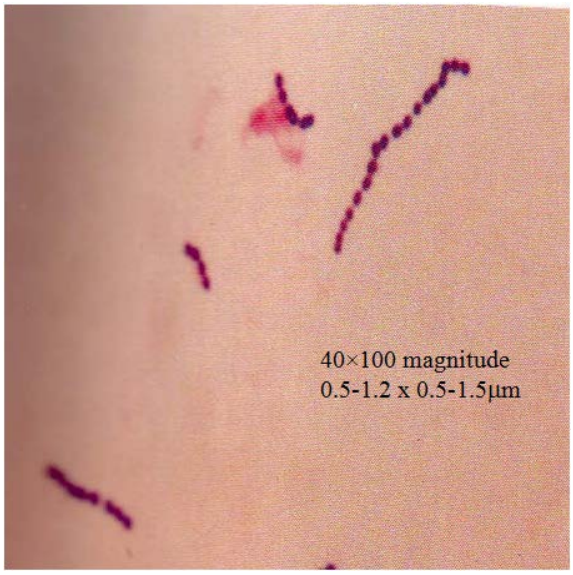

Figure 6. Streptococuss sp. (sample-2, isolates A_{7B})

Based on colony characteristics appeared on Agar media (MacConkey agar media) three bacteria (A_{3A}, A_{7B} and A_{10C}) were isolated. Morphological cultural and biochemical characteristics of these threebacterial isolates were performed for their identification.

Isolation of E. coli O157 from UHT milk studied by De-Boer and Heuvelink, [18], 25 ml of each milk sample was directly added to modified tryptone soy broth supplemented with novobiocin (20 mg/litter). The inoculated broth was incubated at 37°C for 24 h. Aloopful from the incubated broth was streaked onto Telluritte-Cefixime Sorbitol MacConkey agar plate and incubated at 37°C for 24 h. Sorbitol negative colonies (colorless) were picked up and purified then examined Biochemically (tests were performed to confirm E. coli using Gram staining, Catalase test, Indole, Methyl red, Vogese Proskauer test, Nitrate reduction, Urease production, Simon citrate agar, and various sugar fermentation tests) and serologically.

Table 5. Morphological, Biochemical and Cultural Characteristics of the Isolates A_{3A}

Place of collection	:	Senoara Dairy, Chittagong, Bangladesh
Vegetative Cells	:	Spherical or ovoid shape,Cells formed short chain
Cell Size	:	0.5-1.2 x 0.5-1.5µm
Spores	:	Endospores not formed
Gram stain	:	Gram positive
Acid-fast stain	:	Non acid fast
Flagella stain	:	Not done
Motility test	:	Non motile
Agar colonies	:	Circular colonies
Agar slant	:	Echinulate growth
Nutrient broth	:	Flocculent growth
Glucose broth	:	Flocculent growth with turbidity
Catalase activity	:	Catalase negative
Oxidase test	:	Oxidase negative
Deep glucose agar	:	Moderate surface growth within the media
Oxygen relation	:	Facultative anaerobic
Indole test	:	Negative
Nitrate reduction test	:	Negative

Inorganic medium	:	No growth
Citrate medium	:	Negative
Gelatin liquefaction	:	Not liquefied
Hydrogen sulphide (TSI)	:	Produced
Urease test	:	Negative
Milk agar plate	:	Not hydrolyzed
Starch agar plate	:	Negative
Methyl red	:	Positive
V.P. test	:	Negative
Growth at	:	0°C 10°C 20°C 30°C 37°C 40°C + ++ ++ + + + ++ ++
Fermentation	:	L(+) Lactic Acid and no gas form : Glucose, Arabinose, Mannitol, Xylose. No change: Lactose, Sucrose, Starch, Rhamnose, Cellulose.
Identification	:	The morphological, cultural, Biochemica characteristics of isolate A_{3A} was compared with standard description of 'Bergey's Manual of Determinative Bacteriology'-8th Ed. (Buchanan and Gibbons, 1974) and found closely related with the genus *Streptococcus* described by Rosenbach (1984). The isolate was provisionally identified as *Streptococcus lactis.*

Table 6. Morphological, Biochemical and Cultural Characteristics of the Isolates A_{7B}

Place of collection	:	Senoara Dairy, Chittagong, Bangladesh
Vegetative Cells	:	Spherical or ovoid shaped, cells formed, short chain.
Cell Size	:	0.5-1.2 x 0.5-1.5μm
Spores	:	Endospores not formed
Gram stain	:	Gram positive
Acid-fast stain	:	Non acid fast
Flagella stain	:	Not done
Motility test	:	Non motile
Agar colonies	:	Circular colonies
Agar slant	:	Echinulate growth
Nutrient broth	:	Flocculent growth
Glucose broth	:	Flocculent growth with turbidity
Catalase activity	:	Catalase negative
Oxidase test	:	Oxidase negative
Deep glucose agar	:	Moderate surface growth within the media
Oxygen relation	:	Facultative anaerobic
Indole test	:	Negative
Nitrate reduction test	:	Negative
Inorganic medium	:	No growth
Citrate medium	:	Negative
Gelatin liquefaction	:	Not liquefied
Hydrogen sulphide (TSI)	:	Produced
Urease test	:	Negative
Milk agar plate	:	Not hydrolyzed
Starch agar plate	:	Negative
Methyl red	:	Positive
V.P. test	:	Negative
Growth at	:	0°C 10°C 20°C 30°C 37°C 40°C + ++ ++ + + + ++ ++
Fermentation	:	L(+) Lactic Acid and no gas form:Glucose, Arabinose, Mannitol, Xylose. No change: Lactose, Sucrose, Starch, Rhamnose, Cellulose.
Identification	:	The morphological, cultural, Biochemical characteristics of isolate A_{7B} were compared with standard description of 'Bergey's Manual of Determinative Bacteriology'-8th Ed.(Buchanan and Gibbons, 1974) and found closely related with the genus Streptococcus described by Rosenbach (1984).The isolate was provisionally identified as *Streptococcus cremoris.*

Table 7. Morphological, Biochemical and Cultural Characteristics of the Isolates A_{10C}

Place of collection	:	UHT Milk, Chittagong, Bangladesh
Vegetative Cells	:	Cells formed in medium sized rods.
Cell Size	:	Colonies 2-5 mm in dm.
Spores	:	Spores not formed
Gram stain	:	Gram negative
Acid-fast stain	:	Non acid fast
Flagella stain	:	Not done
Motility test	:	Motile
Agar colonies	:	Colonies are found in metallic sheen.
Agar slant	:	Mucoid growth
Nutrient broth	:	Diffuse cloudiness with diffuse growth
Glucose broth	:	Flocculent growth withheavy sediment.
Catalase activity	:	Catalasepositive
Oxidase test	:	Oxidase negative
Deep glucose agar	:	Moderate surface growth within the media
Oxygen relation	:	Facultative anaerobic
Indole test	:	Negative
Nitrate reduction test	:	Positive
Inorganic medium	:	No growth
Citrate medium	:	Negative
Gelatin liquefaction	:	Not liquefied
Hydrogen sulphide (TSI)	:	Negative
Urease test	:	Negative
Milk agar plate	:	Not hydrolyzed
Starch agar plate	:	Hydrolyzed
Methyl red	:	Positive
V.P. test	:	Negative
Growth at	:	0°C 10°C 20°C 30°C 37°C 40°C 45°C + ++ ++ +++ ++ +
Fermentation	:	Gas production is occurred by fermentation.: Glucose, Arabinose, Mannitol, Xylose. No change: Lactose, Sucrose, Starch, Rhamnose, Cellulose.
Identification	:	The morphological, cultural and biochemical characteristics of isolate A_{10C} was compared with standard description of 'Bergey's Manual of Determinative Bacteriology'-8th Ed. (Buchanan and Gibbons, 1974) and found closely related with the genus *Escherichia.* The isolate was provisionally identified as *Escherichia coli.* *Escherichia coli* described by Escherich*et al.,* in 1885.

5. Discussion

In the present study drawn milk samples were collected aseptically then we examined the drawn raw milk samples either it was mastitis contaminated or not by performing Clinical Mastitis Test.

In this test samples were having no mastitis showing appearance of milk contained no thickening and homogenous color according the standard level studied from Los Angeles County Board of Supervisors and we selected our raw milk sample as sample-1 for our research procedure.

Based on our findings, it was used to discourage the consumption of raw milk. The risks of consuming raw milk instead of pasteurized milk are well established in the scientific literature, and in some cases can have severe or even fatal consequences. The potential benefits on the other hand, are still unclear and would benefit from further investigation. We are left with a large uncertainty about the potential benefits of raw milk but with a clear understanding of the microbial hazards from consuming raw milk. Then sample was pasteurized by home pasteurizer in our research laboratory.

After pasteurization the proportions of RM samples were derived another samples, named HPM samples. Enzyme Linked Alkaline Phosphatase Test was used to determine whether the milk pasteurized or not. This test was used to determine that all retail products have been pasteurized properly by examining the products for alkaline phosphatase enzyme, which was normally destroyed during the pasteurization process. Coli form Test was used by our laboratory to measure the quality of the practices used during the plant processing of retail dairy products. Coli forms detected from pasteurized products can often suggest improper pasteurization or post pasteurization contamination. Coli form tests are conducted following pasteurization primarily to detect bacterial recontamination of the milk. Home Pasteurized milk products must have a coli form count less than 10/ml or gram. In these circumstances samples in certified grading due to presence of less than10/ml no. of bacteria. But Ultra Heat Treated milk products must have a coli

form count less than 1/ml or gram where as UHT samples were selected in noncertified grading due to presence of 2/ml No. of bacteria. Raw milk products must have a coli form count less than 100/ml or gram where as raw milk samples were selected in certified grading due to presence of standard No. of bacteria.

Direct Microscopic Countis followed by American Association of Medical Milk Commission for analysis of certified milk. Our research work was followed by an accuracy of measuring the samples that are suitable for counting bacteria of most types of dairy milk. These are present in heat-treated dairy products signifies either inadequate pasteurization or post-pasteurization contamination. In this circumstances RM samples resulted non satisfied grades but after home pasteurization samples was accepted and enriched similar with UHT samples.

Milk and dairy products are generally very rich in nutrients which provide an ideal growth environment for many microorganisms. Therefore, all milk samples are carefully maintained with a temperature of 0.0° to 4.4°C from collection until the samples arrive in our laboratory within 48hrs from the time of collection. The standard plate count (SPC) method is the first test performed on a milk sample after it arrives in our laboratory. This test is used to evaluate the general sanitation of the dairyman's equipment and the overall health of his herd. This method is one of the procedures used throughout the dairy and regulatory enforcement agencies for the determination of quality and the detection of microbial contamination for raw and retail (processed) milk. For a raw milk sample the total bacterial count must be less than or equal to 100,000/ml. For a retail product the total count must be less than or equal to 20,000/ml. In this circumstances RM samples and UHT samples were selected in accepted grading. Based on our review (Table 5) of the scientific evidence, we conclude that drinking raw milk carries an increased risk of food borne illness as compared to drinking pasteurized milk. We identified several articles that detected a relationship between drinking raw milk and reduced allergies among rural children and infants. The underlying cause for this relationship, however, it has not been identified standard level of bacterial colonies in HPM samples.

In graphical presentation (Figure 1 to Figure 3) the linear data was propagated by discrete colonies. The compiled data was resulted with fluctuation of colonies in different procedure from different samples. So it was resembled as designing gram negative, gram positive or coli forms should to isolate for determining contamination or re contamination from samples before and after pasteurization (Figure 1 to Figure 3).

Organisms can be demonstrated by Gram's stained smear. The organisms give positive reaction to this stain and characteristic chain may be seen. The pathogenic strains grow best on serum or enriched media; blood agar was preferred. Colonies were 1mm diameter, round, smooth, dew drop like. Hemolysis may or may not be present. Research protocols verified the absent growth on MacConkey agar and appeared negative reaction to catalase test. Based on colony characteristics, examined isolate A_{3A} that lactose was fermented to lactic acid and other products such as carbon dioxide, ethyl alcohol, acetic acid, etc. The morphological, cultural and biochemical characteristics of isolate was compared with standard description of 'Bergey's Manual of Determinative Bacteriology'-8th Ed. Buchanan and Gibbons, [17] and found closely related with the genus *Streptococcus* described by Rosenbach (1984). Lactose fermented to lactic acid and other products from isolate A_{7B} (Figure 6) but in research lab we made this isolates as *Streptococcus cremoris* by synthesized a viscous polysaccharide material that forms the slime layer or bacterial capsule. The morphological, cultural and biochemical characteristics of isolate was compared with standard description of 'Bergey's Manual of Determinative Bacteriology'-8th Ed. (Buchanan and Gibbons, 1974) and found closely related with the genus Streptococcus described by Rosenbach (1984).

In our research lab we examined colonies 2-3 mm in diameter. On nutrient agar colonies are circular, low convex, smooth, and colorless. In MacConkey agar referred as large pink colored colony. Blood agar was discolored around the growth; reported as haemolysis. Nutrient broth was diffused cloudiness, heavy sediment. MacConkey agar represented the colonies having a metallic sheen, characteristic to *E. coli* (Figure 5). The morphological, cultural and biochemical characteristics of isolate A_{10C} was compared with standard description of 'Bergey's Manual of Determinative Bacteriology'-8th Ed. (Buchanan and Gibbons, 1974) and found closely related with the genus Escherichia. The isolate was provisionally identified as *Escherichia coli. Escherichia coli* were described by Escherich *et al.*, in 1885.

6. Conclusion

Research methodology approached in a comparative study by analyzing these samples and became to a conclusive end with selected harmful bacteriological isolates presence in Pasteurized Milk. Generally, an attempt was made to isolate and identify the causative microorganisms that are responsible for the spoilage of Raw, Pasteurized and UHT milk. So samples derived from different cows and companies were used as test samples to isolate and identify the associated bacteria specified for health risk and human consumption. The results of microbiological assessment in the laboratory and the corresponding questions that were asked to the milk handler and milk servers also suggested that the microbial safety of investigated milk samples depend not only the environmental conditions but also on the personnel hygiene. In this study an attempt was made to isolate the fastidious microorganisms from the raw milk as well as the milk that are pasteurization by using the conventional methods. Nutrient composition of pasteurized milk of four major companies (Farm Fresh, Milk vita, Aarong and Pran) and some raw milk (Senoara Dairy farm) that are not pasteurized as well as their causative organisms responsible for the spoilage were studied.

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