MICROBIAL ANALYSIS OF RAW AND DIFFERENT BRANDS OF PASTEURIZED MILK

A THESIS
BY

MD. AMINUL ISLAM
REGISTRATION NO.: 1105116
SEMESTER: JULY-DECEMBER, 2012
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MASTER OF SCIENCE (M.S.)

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
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HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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DECEMBER, 2012

DEDICATED
TO MY
BELOVED PARENTS

ACKNOWLEDGEMENT

All praises and deepest sense of gratitude be to Almighty Allah, the supreme creator of this universe, who enabled the author to complete this thesis.

The author would like to express his deepest sense of gratitude, sincere appreciation, profound regards and indebtedness to his teacher and research Supervisor, ***Prof. Dr. Md. Mostafizer Rahman****, Dean, Faculty of Veterinary and Animal Science and chairman, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for his scholastic and dynamic guidance, constant inspiration, cordial consistence, affectionate feeling, utmost desire, sympathetic supervision and constructive criticism in all phases of this study and preparing of the manuscript.*

The author finds it a great pleasure in expressing his heartfelt gratitude and immense indebtedness to his Research Co-supervisor ***Prof. Dr. Md. Shahidur Rahman Khan****, Department of Microbiology and Hygiene, Bangladesh Agricultural University(BAU), Mymensingh for his sympathy, kind co-operation, inspiration and constructive criticism for the completion of the research work, and preparation of the thesis.*

The author would like to acknowledge his gratitude and sincere appreciation to respected teachers ***Mir. Rowshan Akte and Md. Fakhruzzaman****, Assistant Professor, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, for their valuable advice, constant help, continuous encouragement and contribution in completing the thesis.*

Thanks are also extended to my friends Khalesur, Abubokor, Mahmudul, Mosiur and Umme kulsum for their encouraging attitude in the study period. The author expresses his thanks to all technicians and office staffs of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for their technical assistance during the research.

Finally, the author is ever indebted to his beloved parents for their endless sacrifices, heartiest blessings and all out support throughout his entire life.

The Author
December, 2012

ABSTRACT

A total of 32 milk samples were collected from 8 sources. Among them three were raw (designated as R1 to R3) and five were pasteurized milk (designated as P1 to P5). Each of the collected samples was investigated during the period from May to December, 2012. Microbiological tests such as viable plate count, coliform plate count, Staphylococcus plate count and Gram's staining were performed to determine the loads of microbes in raw and different brands of pasteurized milk. Total viable counts (TVC) of three raw milk samples, R1, R2 and R3, were 745000 cfu/ml (log 5.87), 1357500 cfu/ml (log 6.13) and 1972500 cfu/ml (log 6.29), respectively. The presence of *Escherichia coli* in the samples of R1, R2, and R3 were 236 cfu/ml (log 2.37), 268 cfu/ml (log 2.42), 940 cfu/ml (log 2.97), respectively, but the presence of *Staphylococcus* were 798 cfu/ml (log 2.90), 590 cfu/ml (log 2.77), 615 cfu/ml (log 2.78), respectively. The average values of TVC (cfu/ml) were 98500 cfu/ml (log 4.99), 68450 cfu/ml (log 4.83), 195000 cfu/ml (log 5.29), 18950 cfu/ml (log 4.27) and 53375 cfu/ml (log 4.72), respectively for the samples of P1, P2, P3, P4, and P5 brands of pasteurized milk, where as Staphylococcal count were 869 cfu/ml (log 2.93), 647 cfu/ml (log 2.81), 443 cfu/ml (log 2.64), 858 cfu/ml (log2.93) and 28 cfu/ml (log1.45), respectively. The coliform counts were 166 cfu/ml (log 2.21), 91 cfu/ml (log1.95), 348 cfu/ml (log 2.54) and 101 cfu/ml (log 2.00) for the samples of P1, P2, P3, and P5, respectively. Coliform were not detected in P4 pasteurized milk sample. Therefore, it was concluded that high counts of bacteria were found in most of the samples. The detection of high counts of *Staphylococcus* coupled with the presence of *E. coli* are of public health significance to consumers.

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LIST OF ABBREVIATIONS AND SYMBOLS

BAU	:	Bangladesh Agricultural University
BGA	:	Brilliant Green Agar
BSTI	:	Bangladesh Standard Testing Institute
CDC	:	Central Disease Centre
CFU	:	Colony forming units
CIP	:	Clean in the Place
E. Coli	:	*Escherichia coli*
e.g.	:	Example
et al.	:	Associated
Etc	:	Etcetera
EU	:	European Union
Fig.	:	Figure
Gm	:	Grams
HACCP	:	Hazard Analysis and Critical Control Point
hrs	:	Hours
HSTU	:	Hajee Mohammad Danesh Science and Technology University
Ib	:	Pound
Kg	:	Kilogram
Ltd	:	Limited
MCA	:	MacConkey Agar
mg	:	Milligram
min	:	Minutes
ml	:	Milliliter
mm	:	Millimeter
No.	:	Number

List of abbreviations and symbols (Continued)

PCA	:	Plate count agar
Prof.	:	Professor
Sec	:	Seconds
SL.	:	Serial
spp.	:	Species
Sq	:	Square
SSOP	:	Standard Sanitation Operating Procedure
TBC	:	Total bacterial counts
TCC	:	Total coliform count
TSC	:	Total Staphylococcus count
TVC	:	Total viable counts
UHT	:	Ultra high temperature
US	:	United State
-	:	Negative
%	:	Percentage
/	:	Per
+	:	Positive
<	:	Less than
>	:	Greater than
µg	:	Microgram
µl	:	Micro liter
^{0}C	:	Degree Celsius

Chapter-1

INTRODUCTION

CHAPTER-1

INTRODUCTION

Milk, considered as nature's single most complete food (O'Mahony, 1988), is the most valuable and regularly consumed foods throughout the world. Like most of the countries, in Bangladesh milk is generally sold in two ways; most cases the farmers bring milk in open pots and sell it directly in the market without any processing and in another case, the milk companies collect milk from the farmers or dairy farms, process it by pasteurization treatment and package the processed milk which is then sold in shops under specific brand name.

Milk is highly vulnerable to bacterial contamination and hence is easily perishable (Kim *et al.*, 1983; Steele *et al.*, 1997). Milk and the dairy products is important sources of food borne pathogens and numerous epidemiological reports have implicated inadequate heat treated milk and raw-milk products as the major factors responsible for illnesses caused by food-borne pathogens (De Buyser *et al.*, 2001; Harrington *et al.*, 2002). So it is necessary that this product to be obtained as cleanest possible, that it would be of the finest condition and not to harm the consumers' health (Dan *et al.*, 2008).

Cross-contamination with pathogenic microorganisms can gain access to milk either by faecal contamination or by direct excretion from the udder into milk. Milk may be contaminated through infected cow having tuberculosis, brucellosis, mastitis and also from human carrier having typhoid fever, diphtheria, dysentery and scarlet fever (Pelczar *et al.*, 1993). As milk is an excellent growth medium for bacteria it can easily be contaminated by many other distinct sources including body of cows, dust

from the air, litter, floor, flies, insects and rodents, water supply, hands and clothes of the milker, utensils, bottles, atmosphere etc (Ensminger *et al.*, 1994; Cousin, 1982). The hygiene and dairy cows' health state, hygiene conditions in the exploitation environment and cleaning means for milk and processing equipment are very important factors that influence the microbial contamination level of raw milk. In the same proportion, the keeping temperatures and lenght of this are important, if not proper, permitting germs to develop. All these factors influence the Total viable count (TVC), Total coliform count (TCC) and bacterial type's existent in the market milk.

In milk, the wide range of microorganisms is frequently reported. Their majority is destroyed during pasteurization but some, like *Pseudomonas florescens* can produce exogenous proteolytic and lipolytic enzymes which are thermostable and capable to alterate the pasteurized milk. Some species and types like *Bacillus, Clostridium, Corynebacterium, Artrobacter, Lactobacillus, Microbacterium, Micrococcus and Streptococcus* can survive pasteurization and can develop at cooling temperatures causing the alteration of contaminated products (Oliver *et al.*, 2005). Pathogens that have been involved in food borne outbreaks associated with the consumption of milk include *Listeria monocytogenes, Salmonella spp., Escherichia coli and Staphylococcus aureus*. The presence of these pathogenic bacteria in milk emerged major public health concerns, especially for these individuals (Ryser, 1998).

Consumers prefer natural, wholesome quality food produced and processed in a sound sanitary condition so that it is free from pathogens and any harmful substances. To meet this consumer's demand, production of quality milk is essential. Milk with normal chemical composition, completely free from harmful toxic substances and low in bacterial counts is

considered as quality milk. In Bangladesh, it is quite rare this perishable product has been received particular attention in hygienic distribution to the consumers (Khan *et al.*, 2008). Non-standardized ways are usually followed during collection, processing and production of final milk for consumption and it is usually supplied to the consumers from the urban and rural areas by milkmen. Moreover, adulteration of milk with water is very common in Bangladesh, these not only causes dilution of milk by reducing the milk solids, but also the great concern for the risk of contamination by microorganisms.

The Bangladesh Standards and Testing Institution oblige various the sanitary requirements for the pasteurized milk (BSTI, 2002). However, no standard is known to be established for the raw and UHT treated milk. In the present studies there is an attempt to find out the occurrence and count of microorganisms in both raw and pasteurized liquid milk offered to the market for human consumption with special emphasis on public health significance. In Bangladesh fake representation of product by producer is pretty common. Thus the microbial quality evaluation of milk is vital. Moreover, the results obtained by this study serve as baseline data will be useful in future studies dealing with pathways of contamination and in the development of HACCP systems for hygienic processing of raw milk.

The present studies is conducted with the following core objectives-

- To investigate the occurrence and load of microorganisms in raw and pasteurized milk.
- To compare the microbial load among different brand of pasteurized milk.

Chapter-II

REVIEW OF LITERATURES

CHAPTER-2

REVIEW OF LITERATURES

The purpose of this chapter is to provide a selective review of the research works accomplished in relation to the present study. Literatures on microbiological quality of raw and pasteurized related to this study have been reviewed under the following headings.

2.1 GROUPS OF ORGANISMS FOUND IN MILK

Hayes *et al.* (2001) isolated and count *Achromobacter spp., Alcaligenes, Chromobacterium spp., and Flavobacterium spp.* in raw and Pasteurized milk.

Nörnberg (2010) isolated *Pseudomonas spp., Aeromonas, Serratia spp., Clostridium spp., Paenibacillus spp., Streptococcus spp. and Staphylococcus spp.* species of bacteria and found their effect on milk quality.

Surhaug and Stepaniak (1997) indentified both Gram's negative (*Pseudomonas spp., Aeromonas, Serratia spp., Achromobacter spp., Alcaligenes, Chromobacterium spp., and Flavobacterium spp.*) and Gram-positive bacteria (*Bacillus spp., Clostridium spp., Paenibacillus spp., Streptococcus spp., Staphylococcus spp.*) bacteria in raw milk.

Gunasekera (1993) reported that psychrotrophic microorganisms are the most important group of microbes present in milk and dairy products. Predominant psychrophilic bacteria he isolated *Aerobacter, Alcaligenes, Achromobacter, Flavobacterium, Micrococci* and *Pseudomonas*. The microbe *Pseudomonas spp.* is considered as the most important psychrotrophs contributing to milk spoilage he also observed.

Moore *et al.* (1947) showed that *Micrococci* were the most predominant among the organisms that were noted to enter the milk from the udder.

Bryan *et al.* (1946) isolated Gram negative rods, *S. 1actis* and *Pseudomonas* species in the milk.

Kennedy and Weiser (1950) reported that the possibility of adaptive mesophilis beginning to grow in milk after 3 -5 days incubation at 10^{0}C thereby being an important factor in building up the psychrophilic microflora.

2.2 SOURCES OF MICROBES IN MILK

Huck *et al.* (2008) observed milk can be polluted by *Mycobacterium bovis, Brucella species, Streptococci and Coxiella burnetti* from infected cattle. They also identified the agents from human sources such as *Salmonella* species, *Shigella* species, *Corynebacterium diphtheria* and *Streptococcus* species can also be presented in milk.

Richard Wallace (2008) reported that the exterior of the cows udder and teats can contribute microorganisms that are naturally associated with the skin of the animal as well as microorganisms that are derived from the environment in which the cow is housed and milked.

Huck *et al.* (2007) reported a wide variety of sources such as workers, infected cows udder, faeces, dust in sheed, milk containers or other equipment can contaminates the milk.

Fromm and Boor (2004) reported the increased CIP cycling times and proper maintenance of filling heads and forming mandrels decreased the amount of contamination and increase the shelf life from nine to 20 days.

Gruetzmacher and Bradley (1999) claimed processing plant include the pasteurizer, the filling heads and the carton forming mandrels for contamination of processed milk. The filling heads and the carton forming mandrels were found to be greater sources of contamination than the pasteurizer.

Rogick and Burgwald (1952) reported that the mesophilic counts were approximately three times greater than the psychrophilic counts in raw milk.

Wayne and Macy (1933) reported an average bacterial count of 659/ml in milk from normal udder and 964/ml.

Harding *et al.* (1921) observed that the bacterial counts on milk drawn aseptically from normal udders were commonly low (25%).

2.3 MICROBIAL COUNT OF RAW AND PASTEURIZED MILK

Melisa Anderson *et al.* (2011) determined the presence and levels of microbes in 20 representative unexpired pasteurized milk samples from six (6) supermarkets from randomly selected supermarkets. The result shown highest colony count of 13 400 cfu/ml and coliform count of 500 CFU/ml.

Hossain *et al.* (2010) examined twelve different liquid market milks of Bangladesh for the enumeration of bacterial count. Results revealed that all the raw milks had high bacterial load which ranged from 1.75×10^6 to 1.22×10^8 cfu/ml. Whereas,The TVBC (total viable bacterial count) of the pasteurized milk samples ranged from 7.5×10^7 to 1.24×10^8 cfu/ml, much higher than that recommended by BSTI. Pasteurized milk didn't contain any coliform, whereas TCC of the other two pasteurized milks were 3.7×10^4 and 1.2×10^6 cfu/ml. The UHT-milks were completely free from any coliform.

Dahal *et al.* (2010) carried out an investigation to evaluate the quality of raw milk measured by total bacterial count (TBC). Results showed a great variability of TBC for the overall study period. The lowest TBC (2.78×10^6) and the highest TBC (13.299×10^6) at two milk collection units revealed nearly fivefold difference. The results of mean TBC at farm (9.03×10^5) and mean TBC at plant (104.71×10^5). The highest number of TBC was 16.5×10^6 cfu/ml.

Shekhar Chandra *et al.* (2010) investigated 60 milk samples from different milk vendors of different areas of the Faizabad district were subjected to different microbial counts and antibiotic sensitivity of bacterial isolates. Range (Mean ± SE) of different microbial counts viz., *coliform, E. coli, faecal Streptococci, Staphylococcus aureus,* yeast and mould, and total viable counts were found as 0.00 - 4.50 (3.00 ± 1.15), 0.00 - 1.20 (0.70 ± 0.46), 1.00 - 2.80 (1.80 ± 0.64), 0.00 - 3.00 (2.10 ± 1.14), 0.00, 2.40 (1.00 ± 0.78), and 3.80 - 7.20 (5.50 ± 0.99) $\log_{10}$ cfu/ml, respectively.

Amico *et al.* (2010) evaluated the overall milk quality and prevalence of 4 target pathogens (*Listeria monocytogenes, Staphylococcus aureus, Salmonella* spp., and *Escherichia coli* O157:H7) in raw milk used for small-scale artisan cheesemaking. Overall, 86% of samples had SPC <10,000 cfu/ml, with 42% <1,000 cfu/ml. Additionally, 68% of samples tested were within pasteurized milk standards for coliform bacteria under the United States' Grade A Pasteurized Milk Ordinance at <10 cfu/mL. Log_{10} SPC and CC did not differ significantly among species. Fourteen of the 21 farms (67%) were positive for *Staphalococcus aureus* were detected in 38% of samples at an average level of 20 cfu/ml.

Lingathurai *et al.* (2009) analyzed sixty samples of raw cow milk for microbiological quality assessment. Microbiological enumeration revealed for the counts of total mesophilic aerobic bacteria, 5.84 log cfu/ml; bacterial endospores, 2.37 log cfu/ml; lactic acid bacteria, 4.46 log cfu/ml; coliforms, 2.76 log cfu/ml; *Escherichia coli*, 1.63 log cfu/ml and *Staphylococcus aureus*, 1.92 log cfu/ml.

Murugan *et al.* (2010) examined sixty pasteurized milk samples from different processing dairies for total viable count. The total viable count (log_{10} cfu per ml) varied between 4.18 ± 0.095 and 4.43 ± 0.081 among nine different processing dairies and it was the lowest in lab-pasteurized samples (3.65 ± 0.078).

Murugan *et al.* (2009) reported the mean aerobic spore count (log_{10} cfu per ml) varied between 3.56 ± 0.051 and 3.87 ± 0.112 among the processing dairies and the lowest values were obtained in lab pasteurized milk samples at 3.09 ± 0.085.

Dan et *al.* (2008) investigated 24 raw milk samples from a milk processing unit. The aerobic mesophillic load of raw milk presented different values, being comprised between 4.24±0.0.12 log cfu/ml and 7.39±0.0.45 log cfu/ml, 38.75% from the total number of milk samples crossing the limit of 6.0 log cfu/ml (10^6 cfu/ml). Regarding the psychrotrophic bacterial load it was noticed that this varried, being situated between 4.65±0.16 log cfu/ml and 7.59±0.45 log cfu/ml.

Khan *et al.* (2008) investigated the microbiological quality of raw milk (total viable count, *Coliform count* and *Staphylococcal count)* from different villages and Bangladesh Agricultural University (BAU) Dairy Farm of Mymensingh District of Bangladesh, during the period from July to November 2007. A total 100 raw milk samples were collected at morning

and evening from BAU dairy farm and surrounding four villages of BAU campus. The average values of TVC/ml were log 5.920, 5.934, 6.007, 6.075 and 6.127 for BAU Dairy Farm, Boira, Shutiakhali, Churkahai and Paglabazar, respectively; coliform count were log 2.501, 2.522, 2.550, 2.620 and 2.619 respectively; Staphylococcal count were log 2.832, 2.812, 2.866, 2.931 and 2.988, respectively.

Karmen *et al.* (2008) reported the total bacterial count higher than 100 000 cfu/ml in 48 (23.6%) out of all tested samples. Its mean value in all milk samples was 4.5 $\log_{10}$ cfu/ml. The mean numbers of coliform bacteria, psychrotrophic micro-organisms, yeasts and moulds together, and coagulase-positive Staphylococci were 2.1 $\log_{10}$ cfu/ml, 3.7 $\log_{10}$ cfu/ml, 2.3 $\log_{10}$ cfu/ml and 1.97 $\log_{10}$ cfu/ml, respectively. The yeasts were present in 95.0% of raw milk samples with the mean concentration of 1.7 $\log_{10}$ cfu/ml. Moulds were found in 63.3% of raw milk samples, their mean concentration was 0.6 $\log_{10}$ cfu/ml. Isolated mould strains belonged to genera *Geotrichum* (51.5%), *Aspergillus* (33.8%), *Mucor* (5.9%), *Fusarium* (2.9%) and *Penicillium* (2.9%).

Chatterjee *et al.* (2006) studied milks ten raw milk samples, the microbial colonies were found to be high in six samples and the colony content was low in rest four samples. In pasteurized milk samples, the colonies were low in seven samples and high in three samples. The methylene blue test performed for raw milk samples showed that out of ten samples, the five samples were poor, two samples were fair, two samples were good and only one sample was found to be an excellent. Out of ten pasteurized samples, nine samples were of good quality and one was found to be excellent. Bacterial colony was found to be opaque and metallic sheen in colour prepared from five raw milk samples, and by biochemical characterization it was identified as *Escherichia coli.*

Revelli *et al.* (2004) investigated a total of 6,998 raw milk samples of bulk tank, result revealed that the total mesophilic aerobic microorganisms count, obtaining a medium value of $1.2 \times 10^5 +/- 2.4 \times 10^5$ cfu/ml that characterizes the zone. The final year of experience, observed a 97% of dairy farms evaluated with averages < or = 1.0×10^5 cfu/ml. Only a 3% it surpasses this limit, not being found establishments with levels over 1.5×10^5 cfu/ml.

Boor et al. (1998) determined bacterial numbers by standard plate count, coliform count, spore-forming psychrotroph count, aerobic spore count, psychrotrophic count, and preliminary incubation count. Paired correlation analyses between the microbiological parameters showed low correlations between test results; no correlation coefficients were > 0.8. The four highest positive correlation coefficients were found between standard plate count and psychrotrophic count (0.7685), psychrotrophic count and preliminary incubation count (0.6648), standard plate count and preliminary incubation count (0.5800), and aerobic spore count and laboratory-pasteurized count (0.5393). All other correlation coefficients were < 0.5. Milk freezing points and acid degree values were determined for all samples. Frequency distributions for these results were also presented.

Adesiyun (1994) investigated bacteriological quality of pre-processed raw milk originating from all 16 milk collections. The mean total aerobic counts for bacteria, *Staphylococcus aureus* and *Escherichia coli* were determined. The total aerobic plate count per ml was generally high for all samples ranging from $5.8 \times 10^5 +/- 3.1 \times 10^5$ to $5.7 \times 10^8 +/- 1.5 \times 10^9$. *S. aureus* was isolated from 478 (94.3%) samples and the mean counts per ml ranged from $1.5 \times 10^5 +/- 1.1 \times 10^5$ to $9.4 \times 10^5 +/- 1.3 \times 10^6$. *E. coli* was isolated from 105 (20.7%) samples and the mean counts per ml ranged from $6.6 \times 10^2 +/- 1.1 \times 10^2$ to $4.0 \times 10^5 +/- 4.6 \times 10^5$.

2.4 MICROBIAL QUALITY OF RAW MILK

Nornberg (2010) observed the quality of the raw milk directly affects the quality of the finished, pasteurized product. Psychrotrophic bacteria, which are able to propagate and metabolize at refrigeration temperatures (0-7°C) are major contaminants in raw milk and can be a common cause of fluid milk spoilage.

Moore (2001) identified psychrotrophic bacteria had become an increasing problem since the introduction of refrigerated raw milk-storage tanks on the dairy farm.

Ezzati *et al.* (2010) reported that Psychrotrophs were capable of producing both proteolytic and lipolytic enzymes during growth at psychrotrophic temperatures, which may be heat-stable and remain active after pasteurization.

Nörnberg (2010) showed that lipolytic enzymes that were produced by some *Pseudomonas spp.* can hydrolyze triglycerides, which are the main lipid component of milk, creating short chain fatty acids or free fatty acids (FFA).

2.5 MICROBIAL QUALITY OF PASTEURIZED MILK

László Varga (2007) reported the hygienic properties of commercially available liquid milks. Although none of the samples tested contained *Salmonella spp.* or *Listeria monocytogens*, approximately 14% of them failed to meet the legal requirements in terms of overall hygienic quality.

FDA (2009) reported that pasteurization kills 99.999% of pathogens. Endospores formed by PGPE bacteria, which are present at any given time during the bacterial growth cycle, can survive pasteurization and spoil the milk during its shelf life.

Huck *et al.* (2008) investigated that PGPE bacteria such as Bacillus are especially difficult to control as they can be found on the dairy farm as well as in the processing plant.

Burgess *et al.* (2010) reported PGPE bacteria have the ability to form bio-films on stainless steel equipment and continually contaminate milk passing through pipelines and equipment.

2.6 STANDARD OF TOTAL VIABLE COUNT AND COLIFORM COUNT IN MILK IN DIFFERENT COUNTRY/REGION

Region	Total Viable Count	Coliform Count	Grade
The USA[1]	200,000	Nil	A
	1,000,000	10	B
	Not Limit	100	C
The EU[2]	<100,000	Nil	-
Denmark[3]	<30,000	Nil	Extra Superior Quality
	30,000-100,000	Nil	Satisfactory Quality
	100,000-300,000	100	Less Satisfactory Quality
	300,000-800,000	-	Non-Satisfactory Quality
	>800,000	-	Very unsatisfactory
India[4]	Not excceding 200,000	Nil	Very good
	Between 200,000-1,000,000		Good
	Between 1,000,000-5,000,000		Fair
	Over 5,000,000		Poor
Foster et al., 1979; Park et al., 1995; Des et al., 2005			

Pamela *et al.* (2002) showed that in pasteurized milk, the SPC must be under 20,000 cfu/ml for the US standard and between 5000-50,000 cfu/ml for European Union (EU) standard.

Henningson (1961) used a standard of 50, 000/ml total count and 100/ml of coliform as the basis to determine the suitability of the coliform count as an index of quality. He reported that there was little relationship between the two counts.

BSTI (2002) showed total viable count of pasteurized milk is 2×10^4 cfu/ml and Coliform count less then 10 /ml. No standard for coliform and *Staphylococcus* count for raw milk.

2.7 PASTEURIZATION OF LIQUID MILK

Redmond (2005) defines pasteurization, as a process of heating a liquid, particularly milk, to a temperature between 55 ^{0}c and 70 ^{0}c, to destroy harmful bacteria without materially changing the composition, flavor, or nutritive value of the liquid. The main aim of pasteurization of milk is to reduce microbial load.

Gunasekera (2002) reported the milk pasteurization was introduced as a public health measure in order to destroy human pathogens and to eliminate or reduce the activities of spoilage microorganisms.

Smith *et al.* (1981) reported pasteurization can prevent diseases include tuberculosis, brucellosis, diphtheria, scarlet fever, and Q-fever; it also kills the harmful bacteria *Salmonella, Listeria, Yersinia, Campylobacter, Staphylococcus aureus*, and *Escherichia coli* among the other.

2.8 MICROBIAL QUALITY OF MILK AND ITS PUBLIC HEALTH SIGNIFICANCE

Todar (2008) observed coliform bacteria are normal inhabitants of the large intestine. The presence of these organisms in milk therefore indicates fecal contamination.

Oliver *et al.* (2005) reported that products derived from milk of dairy animal can harbor a variety of microorganisms and can be important sources of foodborne pathogens.

Leloir *et al.* (2003) evaluated the *Staphylococcus aureus* as one of the important causes of food-borne diseases in humans, but was commonly associated with intoxications due to its ability to produce a variety of potent enterotoxins Processing of milk by pasteurization has been scientifically proven to be at least 90% effective in eliminating harmful bacteria in milk. Non pasteurized, raw milk, according to the Centers for Disease Control (CDC), was responsible for 86 reported food poisoning outbreaks between 1998 and 2008, resulting in 1,676 illnesses, 191 hospitalizations, and two deaths. Improperly handled raw milk is responsible for nearly three times more hospitalizations than any other foodborne disease outbreak .

Quinn *et al.* (2002) identified that *Escherichia coli* as the most common contaminant of raw and processed milk. He also found *E. coli* as a commensal micro-organism of the intestines of animals and humans but its recovery in food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains.

Soomro *et al.* (2002) observed enteropathogenic *E. coli* strains can cause severe diarrhea and vomiting in infants and young children, while toxigenic strains like *E. coli* O: 157:H7 cause haemolytic and uremic syndrome.

Prescott *et al.* (1999) reported campylobacter jejuni was considered as a leading cause of acute bacterial gastroenteritis in humans. As little as 10 of these bacterial cells were lead to the onset of diarrhea and it was transmitted by raw milk.

Mowlem (1988) showed that salmonellosis, tuberculosis, brucellosis, listeriosis, Q-fever, toxoplasmosis, streptococcal infection, staphylococcal infection and campylobacter infection usually obtained from milk.

Chapter-III

MATERIALS AND METHODS

CHAPTER-3

MATERIALS AND METHODS

The present studies were conducted during the period from May to December, 2012 in the Microbiology laboratory of the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The detailed outline about the materials and methods used are given below.

3.1 MATERIALS

3.1.1 SAMPLE

Sources of the population in this study were raw and pasteurized milk presented for sale at the daily market. All pasteurized milk were of specific brand name where as raw were collected from local market, dairy farm and different milk collection point at Dinajpur District.

3.1.2 MEDIA FOR CULTURE

3.1.2.1 PLATE COUNT AGAR

Plate count agar (Merck Specialties Private Litd) was used for the enumeration (cfu/ml) of Total viable count (TVC) of Bacteria.

3.1.2.2 STAPHYLOCOCCUS AGAR NO. 110

Staphylococcus agar no. 110 (Titan Biothech Ltd. India) was used for the enumeration (cfu/ml) of *Staphylococci* it act as a selective medium for Staphylococci which causes enhancement of the growth of *Staphylococcus* while inhibiting the growth of other contaminating organisms and shows typical colony characters.

3.1.2.3 MAC CONKEY AGAR (MCA)

Mac conkey (MCA) medium (Merck Specialties Private Ltd. India) was used for the enumeration (cfu/ml) of coliform organism in milk samples.

3.1.3 REAGENTS USED FOR THE STUDY

Gram's staining reagent:

- Crystal violet,
- Gram's iodine,
- Acetone and
- Safranine

3.1.4 LABORATORY PREPARATION

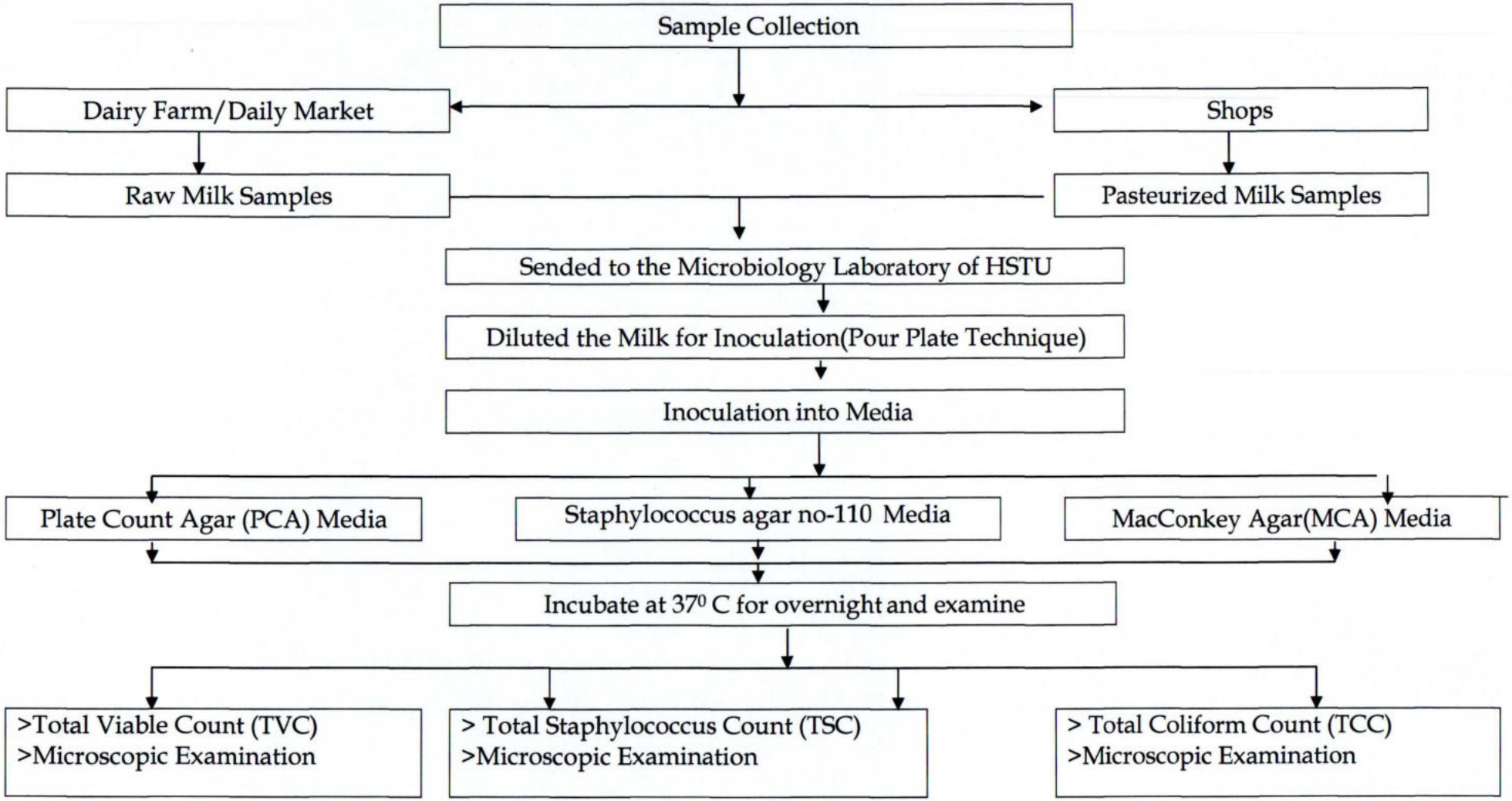
All items of glassware including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides, vials and petridish were soaked in a household dishwashing detergent solution ('Trix' Recket and Colman Bangladesh Ltd.) for overnight, contaminated glassware were disinfected in 2% sodium hypochloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 121 ^{0}C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven over at 50 ^{0}C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50 ^{0}C for future use.

3.1.5 INSTRUMENT AND APPLIANCES

Digital colony counter, electric water bath, phase contrast microscope, digital pH meter, milk collection tubes, cotton, hand gloves, micropipette (1 ml, 500 µl, 10-20 µl), glass slides, eppendorf tube, magnifying glass, marker pen, ice-box, spirit lamp, cover slips, test tube and rack, autoclave, refrigerator, conical flask, etc were used as per necessary.

3.2 METHODS

3.2.1 DESIGN OF THE EXPERIMENT



3.2.2 SOURCES AND COLLECTION OF SAMPLES

Sampling was done by random selecting the shops. Heat treated brand milks (pasteurized) were purchased from different confectioneries where as the raw milks were purchased from a local daily market or dairy farm and milk collection point at dinajpur district.

A total of 32 milk samples were collected from 8 sources, three raw milk (designated as R1 to R3). Of the remaining, five (P1 to P5) were pasteurized milk each from different brands. All the samples were collected following standard sampling procedure and immediately transported to the laboratory of department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University (HSTU); Dinajpur-5200 for microbiological examination.

3.2.3 PREPARATION AND DILUTION OF THE SAMPLE

To obtain countable plate with individual colonies the samples were diluted using simple 1/10 dilutions. For a 1/10 dilution 1 part sample was added to 9 parts diluents (Distilled water). The known amount of a sample was added by pipette to a known amount of diluent the resulting suspension then thoroughly mixed. A series of dilutions was made as desired, and from one or more of these dilutions, known amounts were inoculated in an agar by pour plate techniques.

3.2.4 PREPARATION OF CULTURE MEDIA

3.2.4.1 PLATE COUNT AGAR MEDIA

1.75 grams of Plate Count Agar (MSP-Ltd, India) were suspended in 100 ml of distilled water taken in a flask, heated to boiling to dissolve the medium completely and then sterilized by autoclave at 121°C at under 15 pounds

pressure per square inch for 15 minutes (1.2 kg/cm^2). Approximately 20ml of medium was taken in test tube and the test tube containing liquid medium was placed into electric water bath at the temperature of 40 to 45^{0}C to prevent solidification before pour onto petridish.

3.2.4.2 STAPHYLOCOCCUS AGAR NO-110 MEDIA

14.96 grams of Staphylococcus Agar No-110 media (Titan Biotech Ltd., India) were suspended in 100 ml of distilled water taken in a flask, heated to boiling to dissolve the medium completely and then sterilized by autoclave at 121°C at under 15 pounds pressure per square inch for 15 minutes (1.2 kg/cm^2). Approximate 20ml of medium was taken in test tube and the test tube containing liquid medium was placed into electric water bath at the temperature of 40^{0}C to 45^{0}C to prevent solidification before pour onto petridish.

3.2.4.3 MAC CONKEY AGAR MEDIA

5 grams of ,Bacto-MacConkey Agar (Hi Media, India) were suspended in 100 ml of distilled water taken in a flask, heated to boiling to dissolve the medium completely and then sterilized by autoclave at 121°C at under 15 pounds pressure per square inch for 15 minutes (1.2 kg/cm^2). Approximately 20 ml of medium was taken in test tube and the test tube containing liquid medium was placed into electric water bath at the temperature of 45^{0}C to prevent solidification before pour on to petridish.

3.2.5 MICROBIOLOGICAL EXAMINATION OF SAMPLES

The forgoing prepared milk sample were bacteriologically examined in order to determine the total viable count (TVC), total staphylococcus count (TSC) as well as total colliform count (TCC). Microbiological tests like total viable bacterial count (TVC), total coliform count (TCC), total *Staphyloccus*

spp. count (TSC), were performed on plate count agar (PCA), MacConkey agar(MCA) and Staphylococcus agar no-110 medium respectively. Plates exhibiting colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the TVC. The TVC was expressed as the number of organism of colony forming units per ml (CFU/ml) of samples according to ISO (1995). Gram staining was performed to examine the microbes for identification and different morphological characteristics of the isolated colonies.

3.2.5.1 TOTAL VIABLE COUNT (TVC)

The total viable counts (TVC) of raw and processed milk were determined using the pour plate method which was carried out as described by Quinn *et al.* (2002). Briefly, 1 ml of each of ten-fold dilutions of the sample (raw milk or processed milk) was added onto sterile petri dishes in duplicate and was mixed with 30 ml of molten plate count agar (PCA) (Merck Specialties Private Ltd, India) cooled to approximately 45 ^{0}C. The set agars were incubated aerobically at 37 ^{0}C for 48 h.

3.2.5.2 TOTAL COLIFORM COUNT (TCC)

In the TCC (cfu/ml), the procedure was the same as the standard plate count. But approximately 15 ml of MacConkey agar (MCA) was then added to the labeled sterile petridish and the milk samples were mixed thoroughly and uniformly with the agar. The agar was allowed to be solidified and incubated. A negative control was done using MCA. The plates were then placed on a colony counter and the colonies are counted.

3.2.5.3 TOTAL STAPHYLOCOCCUS COUNT (TSC)

To enumerate *Staphylococcus spp.* in milk, dilutions were prepared for TVBC determination were plated (pour plate techniques) on Staphylococcus agar no.-110 (Manitol Salt Agar) plates. All inoculated plates were incubated at 37°C for 48 h. Characteristic golden yellow colonies were counted and expressed as colony forming units (cfu) of *Staphyloccus spp* per ml.

3.2.5 GRAM'S STAINING

The representative bacterial colonies were characterized morphologically using Gram's stain according to the method described by Cowan (1985). A small colony was picked up with a bacteriological loop, smeared on separate glass slide and fixed by gentle heating. Crystal violate was then applied on each smear to stain for two minutes and then washed with running water. Few drops of Gram's iodine were then added to stain for 1 min. Alcohol act as mordent was then added (act as decolonization) for few seconds. After washing with water, safranine was added as counter stain and allowed to stain for 2 minutes. The slides were then washed with water, blotted and dried in air. Finally slide was examined under microscope with high power objective using immersion oil.

3.2.6 STATISTICAL ANALYSIS

All the data obtained during the study were analyzed statistically to find out the P-value. The P-value and the values in average was determined by ANOVA.

Chapter-IV

RESULTS

CHAPTER-4

RESULTS

A total of 32 liquid milk samples, 12 raw milk samples from dairy farm, chilling center and local market denoted as R1, R2 and R3, respectively with 4 in each case and rest 20 pasteurized milk samples from different shops under specific brand name denoted as P1, P2, P3, P4 and P5 with 4 in each case too, were collected. The liquid milk samples were subjected to bacteriology laboratory of Hajee Mohammad Danesh Science and Technology University (HSTU) to determine the microbial load. The results of bacterial distribution in the collected samples are as follows.

4.1 TOTAL VIABLE COUNT

Total Viable Count (TVC) of bacteria was carried out on plate count agar media using pour plate techniques. The results, presented in Table- 1, showed that the average TVC (cfu/ml) were 745000 (log5.87), 1357500 (log 6.13) and 1972500 (log 6.29) for raw milks collected from different sources, R1, R2 and R3, respectively. The counts for pasteurized milk samples, P1, P2, P3, P4 and P5 were 98500 (log 4.99), 68450 (log 4.83), 195000 (log 5.29), 18950 (log 4.27) and 53375 (log 4.72), respectively.

4.2 COLIFORMS COUNT

The average measures of TCC (cfu/ml) of milk samples were 236 (log 2.37), 268 (log 2.42) and 940(log 2.97) for raw milks collected from different sources, R1, R2 and R3, respectively (Table-1). The results for pasteurized brand milks, P1, P2, P3, and P5 were 166 (log2.21), 91 (log 1.9), 348 (log 2.54) and 101 (log 2.00). Brand P4 had no colonies for coliform organism.

4.3 STAPHYLOCOCCUS COUNT

Staphylococcus agar no-110 medium was used for the enumeration of total staphylococcal count (TSC) in the milk samples. In the raw milks the TSC (cfu/ml) were 798 (log 2.90), 590 (log 2.77) and 615 (log 2.78) for R1, R2 and R3, respectively. The values of TSC (cfu/ml) of pasteurized milks were 869 (log 2.93), 647 (log 2.81), 443 (log 2.64), 856 (log 2.93) and 28 (log 1.44) for P1, P2, P3, P4 and P5.

4.4 RESULT ON GRAM'S STAINING

Gram's stained smears from plate count agar; MacConkey agar and Staphylococcus agar no-110 Medium were examined microscopically which revealed the following results:

- Smear from PCA: Presence of both Gram's positive and Gram's negative organism with different arrangements (Plate- 3).
- Smear from MCA: Revealed Gram's negative, pink color, mostly rood shape organism (Plate- 4).
- Smear from Staphylococcus agar no-110: Showed Gram's positive, violet color, short cocco-bacili or rod and arranged in bundles and singly also (Plate- 5).

Table 1. The average counts of TVC, TCC and TSC in raw and different brands of pasteurized milk

	Sources of Milks	*Numbers of Tested Sample*	**Total Viable Count/ml**		**Total Coliform Count/ml**		**Total Staphylococcus count/ml**	
			cfu/ml	*Log10*	*cfu/ml*	*Log10*	*cfu/ml*	*Log10*
	R1	4	745000	5.87215627	235.5	2.37199091	797.5	2.901730692
	R2	4	1357500	6.13273984	268	2.42813479	590	2.770852012
	R3	4	1972500	6.29501701	940	2.97312785	615	2.788875116
P-value			**0.00645**		**6.14**		**0.061**	
	P1	4	98500	4.99343623	165.5	2.218798	868.75	2.938894818
	P2	4	68450	4.83537345	91	1.95904139	646.75	2.810736437
	P3	4	195000	5.29003461	348	2.54157924	442.75	2.64615857
	P4	4	18950	4.27760921	0		857.5	2.933234129
	P5	4	53375	4.72733789	101	2.00432137	28	1.447158031
P-value			**0.0047**		**1.164**		**1.493**	

Table 2. The ratio between TVC and TCC in raw and different brands of pasteurized milk.

Sources of Milks	*Numbers of Tested Sample*	TVC/ml *cfu/ml*	TCC/ml *cfu/ml*	Ratio-TCC:TVC
R1	4	745000	235.5	1:3163.481953
R2	4	1357500	268	1:5065.298507
R3	4	1972500	940	1:2098.404255
P1	4	98500	165.5	1:595.1661631
P2	4	68450	91	1:752.1978022
P3	4	195000	348	1:560.3448276
P4	4	18950	0	-
P5	4	53375	101	1:528.4653465

Table 3. The Ratio between TVC and TSC in raw and different brands of Pasteurized milk.

Sources of Milks	*Numbers of Tested Sample*	T VC/ml *cfu/ml*	TSC/ml *cfu/ml*	Ration-TSC:TVC
R1	4	745000	797.5	1:934.169279
R2	4	1357500	590	1:2300.847458
R3	4	1972500	615	1:3207.317073
P1	4	98500	868.75	1:113.381295
P2	4	68450	646.75	1:105.8368767
P3	4	195000	442.75	1:440.4291361
P4	4	18950	857.5	1:22.09912536
P5	4	53375	28	1:1906.25

Figure-2: Comparison of TVC of best quality raw and different brands of Pasteurized milk

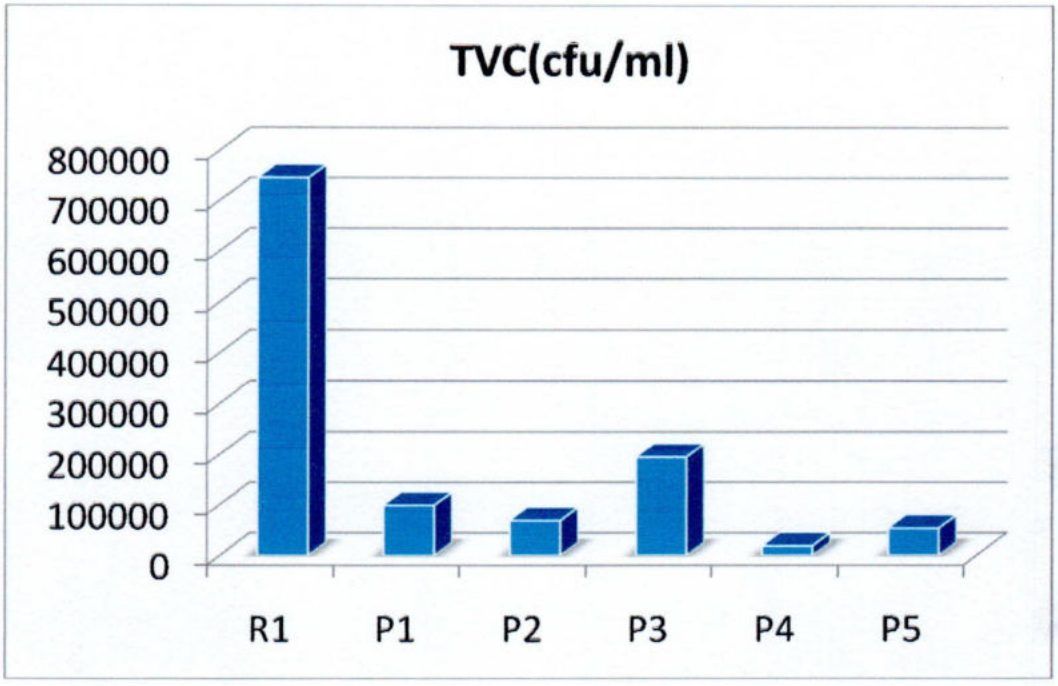


Figure-3: Comparison of TCC of best quality raw and different brands of Pasteurized milk

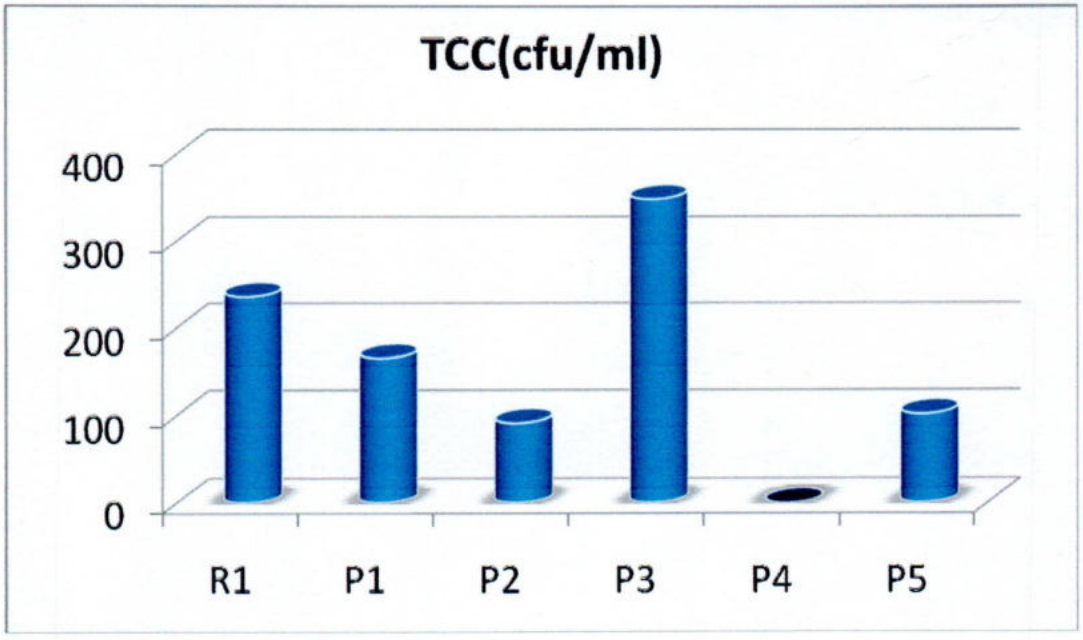
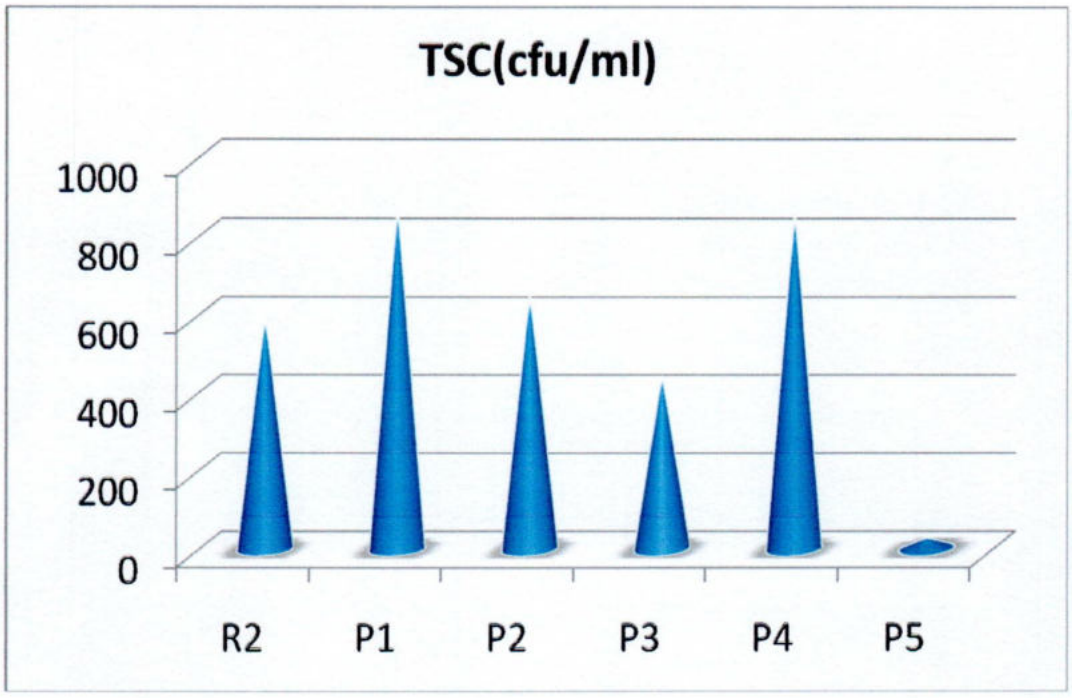


Figure-4: Comparison of TSC of best quality raw and different brands of Pasteurized milk



4.5. RESULT ON PHOTOFOCUS

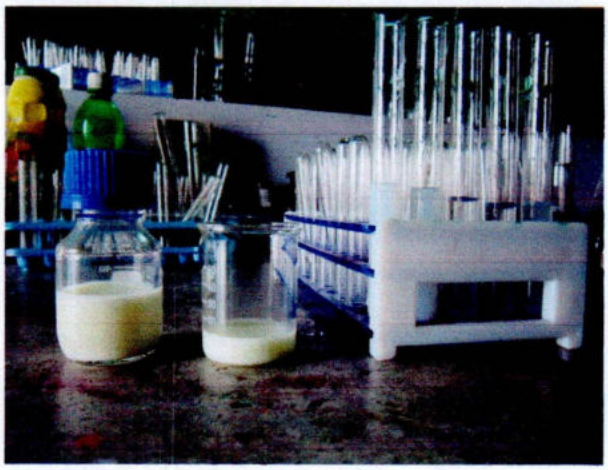


Plate 1: Preparation of dilution of the milk

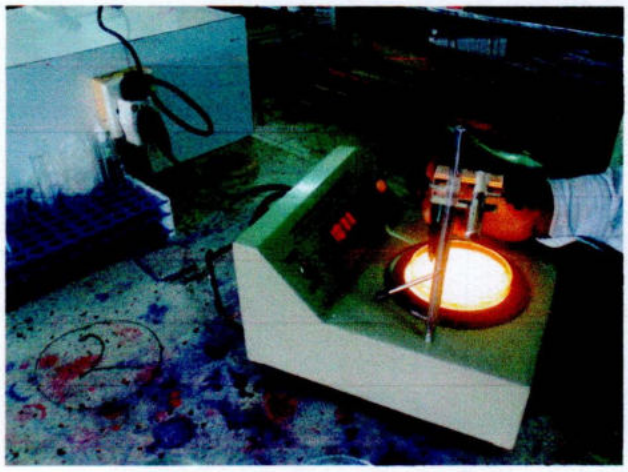


Plate 2: Colony counting

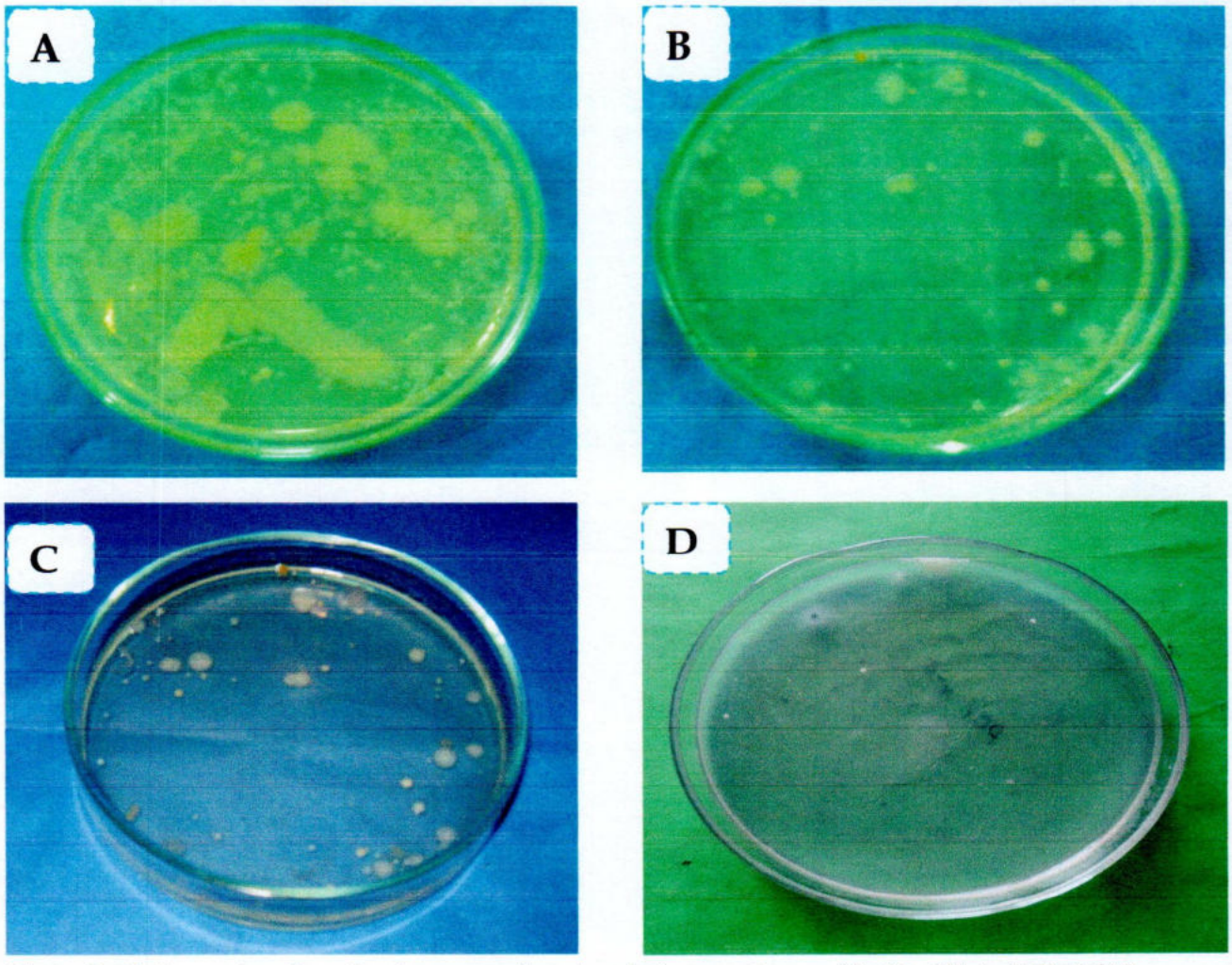


Plate 3: Colony density of microorganism in plate count agar (**A.** Undiluted, **B.** 1/10 dilution, **C.** 1/100 dilution and **D.** 1/1000).

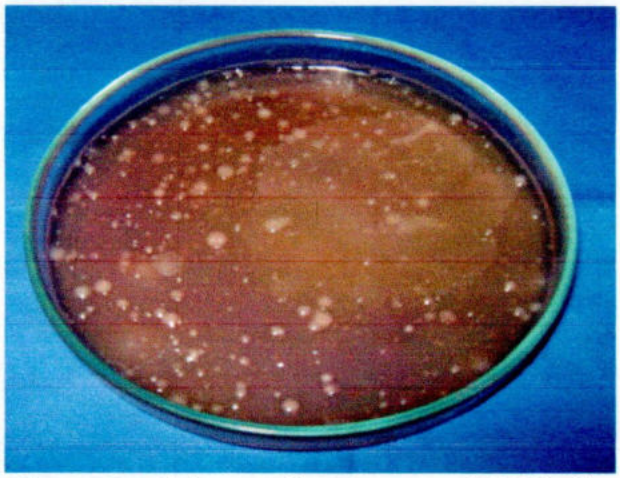


Plate 4: Colony density on MCA for coliform count.

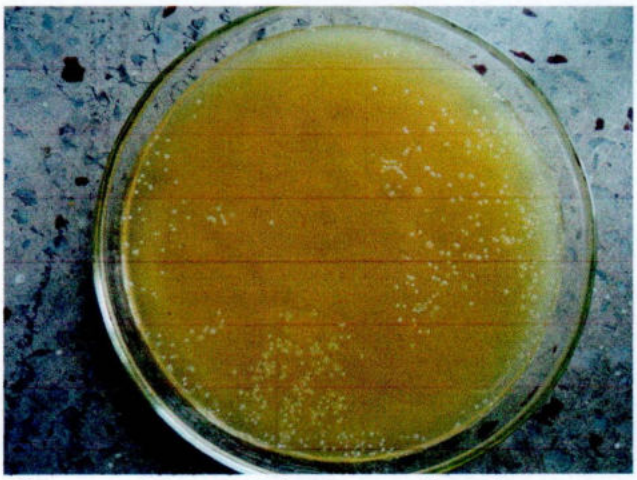


Plate 5: Colony density on Staphylococcus agar no.110 for *Staphylococcus* count.

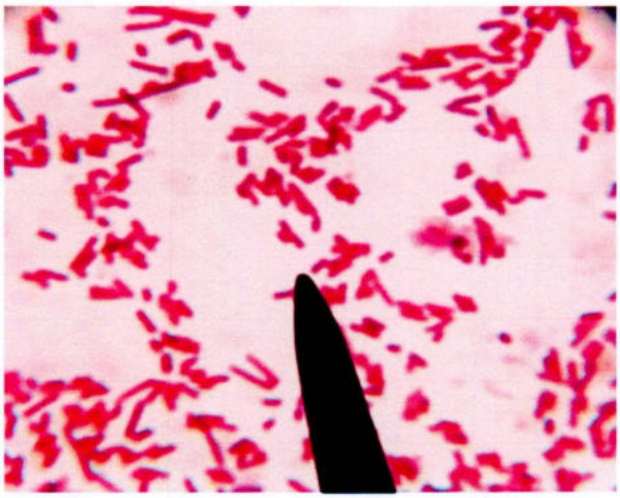


Plate 6: Smear from MCA showing Gram negative small rod-shape, arranged in single or pair (Gram's staining)

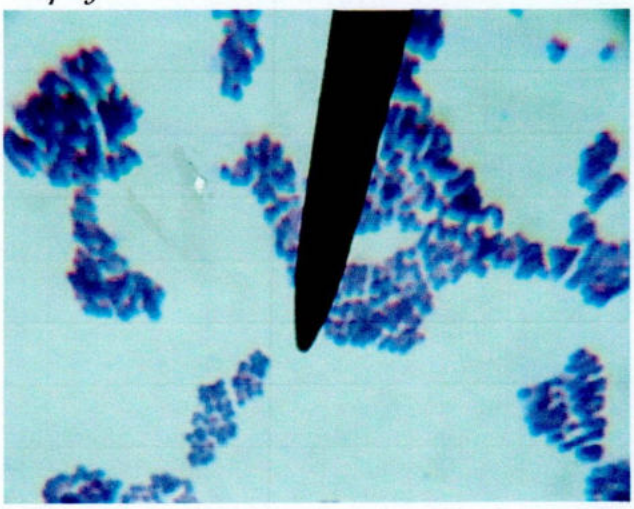


Plate 7: Microscopic feature from Staphylococci agar no-110 showing gram positive, cocci shape, grape like cluster.

Chapter-V

DISCUSSION

CHAPTER-5

DISCUSSION

The present investigation on microbial analysis of raw and different brands of pasteurized milk was conducted from May to December, 2012. Bacteriological investigations were evaluated from 32 milk samples obtained from two important sources viz., raw and pasteurized milk. All the raw milk samples were collected from the different dairy farms, chilling centre and local market at Dinajpur district of Bangladesh where as brand milk from different confectioneries.

In this study PCA, MCA and Staphyloccocus agar no-110 were used for the enumeration of bacteria from milks were in agreement with other workers (Mhone *et al.*, 2011; Hossain *et al.*, 2010). The present study revealed that the total viable counts (TVC) for raw milk were ranges from 74500 cfu/ml to 1972500 cfu/ml , with an average of 1358333 cfu/ml (Table-2) which is corresponding to the findings of Dan (2008). The average count (cfu/ml) for pasteurized milk was observed in samples belong to P-1=98500, P-2= 68450, P-3= 195000, P-4=18950 and P-5=53375 (Table -1). It was evident from the result that the average TVC count was nearly matched to Lingathorni (2009). Average counts for *E. coli* in raw milk samples were 481 cfu/ml where as in pasteurized milk *E. coli* counts for P-1,P-2,P-3,P-5 were 166 cfu/ml, 91 cfu/ml 348 cfu/ml and 101 cfu/ml, respectively. Total Staphylococcal count in the raw milks ranged from 590 to 798 cfu/ml. These counts were less than the findings of Ghose and Maharajan (2002) where the mean Staphylococcal counts were $4.7x10^6$ cfu/ml in raw milk but higher than that of Srairi et al. (2006) where TSC ranged from <30 cfu/ml.

In contrast to different recommended standard for milk foods, sanitary condition of milks sample used for this study were not satisfactory. United State standards recommended, each ml of raw milk for pasteurization must have less than $3x10^5$ cfu/ml (Coast et al., 2004; Reinemann et al., 1999; Jayarao et al., 2001) Unfortunately, the results presented in Table-1 showed that the TVC ranged from 745000 cfu/ml to1972500 cfu/ml is almost far greater than the United State standard.

The bacteria count in the pasteurized milk samples ranged from 18950 to 195000 cfu mL^{-1}, much higher than that recommended by BSTI and USPHS, i.e., not exceeding 20,000 cfu/ml (BSTI, 2002; Jay, 2003). The reason for high bacteria count in the pasteurized milks may include defective pasteurization machinery, survival of pasteurization and post-pasteurized contamination such as poor processing and handling conditions and/or poor worker hygiene.

Coliforms are considered as indicator organisms because their presence in food indicates some form of contamination. The coliforms standards for Grade 'A' pasteurized milk and milk products should not be over 10/ml (BSTI, 2000) and for certified pasteurized milk should not be over 1/ml (Frazier and westoff, 1958, James 1978). The present investigation however reported significantly high coliforms count than that recommended by Frazier *et al. (1958)* and BSTI (2002). *E. coli* causes severe diarrhea in newborns and adolescents and originates from mastitis (Kornalijnslijper et al., 2004). However, the raw and pasteurized milk of this study are not only top grade but also some kinds of them are unusable.

In this study, the results of distribution of organism in liquid milk indicated that the microbial factors are an important indicator for grading of milk either it safe for consumption or not. The hygienic standard of the raw milk was very poor whereas, pasteurized milks of few brand shows tolerable level of

microbial load. The government therefore should conduct frequent inspection of the marketed milks to check whether they meet the minimum legal standards and should monitor the overall hygienic condition surrounding the production and handling of milk. Realistic standards for the raw milks need to be revised and appropriate training should be given to the raw milk producers in hygienic handling of milk.

In consideration of comparative figure of microbial load in the best quality raw milk to different brands of Pasteurized milk, all pasteurized milk contained lower TVC count (Figure-2). For TCC the P-3 brands pasteurized milk contain higher count than the best quality raw milk (Figure-3). In case of *Staphylococcus* count surprisingly P-1, P-2, P-4 contain high bacteria count than raw milk sample (Figure-4).

Chapter-VI

SUMMARY AND CONCLUSIONS

CHAPTER-6

SUMMARY AND CONCLUSIONS

It is concluded from the present study that microbial loads of raw milk are not satisfactory. Therefore, it could be assume that the handler of raw milk do not maintain good personal hygiene. All most all brands of pasteurized milk tested in this study are of low quality based on BSTI standard. Since, packed liquid milk is consumed mainly by children in Bangladesh, therefore, a Standard Sanitation Operating Procedure (SSOP) should be maintained, which is a prerequisite program of Hazard Analysis and Critical Control Point (HACCP), in order to minimize the risk of contamination for safety purpose.

In the context of this study, it may be concluded that,

i. High counts of bacteria in all raw milk samples indicate the low quality from the sanitary point of view.
ii. No brands other than P4 are acceptable based on BSTI (2002) standard.
iii. The presence of *E. coli* and *Staphylococcus* spp. in most of the samples are of public health concern.

Chapter-VII

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CHAPTER-7

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APPENDICES

Plate count agar

Ingredients	g/L
Casein enzyme hydrolysate	5.00
Yeast extract	2.50
Glucose anhydrous	1.00
Agar agar	9.00

MacConkey agar

Ingredients	g/L
Peptone	17.0
Protease peptone	3.0
Lactose	10
Bile salt	1.5
Sodium chloride	5.0
Agar	13.5
Neutral red	0.03
Crystal violet	0.001

Staphylococcus agar no. 110

Ingredients	g/L
Agar	15.00
Dipotassium phosphate	5.00
Sodium chloride	75.00
Lactose	2.00
Mannitol	10.0
Gelatin	3 0.00

Appendices (Continued)

Yeast extract	2.50
Casein enzyme hydroysate	10.00
Distilled water upto	1000 ml

Gram's stain solutions

Ingredients	**g/L**
a. Stock crystal violet	
Crystal violet	10 gm
Ethyl alcohol	1000 ml
b. Stock oxalate	
Ammonium oxalate	1 gm
Distilled water upto	1000 ml

Crystal violet working solution: 20 ml of solution no. 1 mixed with 80 ml of solution no. 2. Additional dilution was made when desired.

c. Lugol's iodine solution	
Iodine crystal	1 gm
Potassium iodide	2 gm

Iodine and potassium iodide were dissolved completely in 10 ml of distilled water, then was added to distilled water to make 300 ml and was stored in amber bottle.

d. Ethyl alcohol	250ml
e. Acetone	250ml
f. Counter stain	
Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml