Nutrition Status and Microbial Hazards in the Milk Supplied at Different Hospitals of Chattogram Metropolitan Area

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Applied Human Nutrition and Dietetics

Department of Applied Food Science and Nutrition

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Statement of Candidate

I hereby declare that I am the sole author of the thesis. I, Afra Binte Iftekhar, declare that this thesis is submitted in fulfillment of the requirements for the Degree of Master of Science (MS) in Applied Human Nutrition and Dietetics, Department of Applied Food Science and Nutrition, Faculty of Food Science & Technology, Chattogram Veterinary and Animal Sciences University. It is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

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The Author

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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The Author

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DEDICATED TO MY

RESPECTED PARENTS,

AND YOUNGER BROTHER

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LIST OF ABBREVIATIONS

Abbreviations	Elaborations
%	Percentage
<	Less than
>	Greater than
°C	Degree Celsius
°F	Degree Fahrenheit
°L	Lactometer Degree
µl	Microlitre
0.1N	0.1 Normal
ANOVA	Analysis of Variance
BDS	Bangladesh Standards
BSTI	Bangladesh Standards and Testing Institution
Conc.	Concentration
CLR	Corrected Lactometer Reading
CFU	Colony Forming Unit
CI	Confidence Interval
CMA	Chattogram Metropolitan Area
CVASU	Chattogram Veterinary and Animal Sciences University
E. coli	*Escherichia coli*
e.g.	As Example
et al.	And his/her associates
etc.	Etecetera
FAO	Food and Agricultural Organization
gm	Gram
govt.	Government
hrs.	Hours
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
LR	Lactometer Reading

Abbreviations	Elaborations
i.e.,	That is
mg	Milligram
ml	Milliliter
mins	Minutes
MR	Methyl Red
PRTC	Poultry Research and Training Centre
rpm	Rotation Per Minute
S. aureus	*Staphylococcus aureus*
SNF	Solids- Not-Fat
SPSS	Statistical Package for the Social Sciences
TS	Total Solids
TSI	Triple Sugar Iron
Temp.	Temperature
TVC	Total Viable Bacterial Count
TCC	Total Coliform Count

Abstract

The present study was undertaken to investigate the quality of milk supplied to the patients by the different hospitals in Chattogram Metropolitan Area (CMA), Bangladesh. Three types of milk were supplied to the patients admitted in different hospitals at CMA. In context of the objectives of the study, a total of 20 samples were collected from 09(nine) different government and private hospitals of CMA to analyze the physical (specific gravity), nutritional (percentage of butter fat, protein, solids-not-fat and total solids), chemical (titrable acidity), added preservatives and adulteration status) and microbial (total viable count, total coliform count, *E. coli* and *Staphylococcus aureus*) parameters to evaluate the quality of the collected milk samples. The lowest and the highest specific gravity were 1.025±0.0012 and 1.03±0.0008 in raw and reconstituted milk respectively. In case of nutritional quality of milk, the highest average percentage of fat, solids-not-fat, total solids were 2.4±0.208, 9.30±0.219 and 11.3±0.354 respectively but the lowest protein% (3.92±0.245) was also found in reconstituted milk samples. The study also reveals that the lowest fat and total solids content were found 1.1±0.281 and 8.44±0.274 percent respectively in raw milk. The highest and the lowest titrable acidity % were 0.18±0.011 and 0.11±0.009 in raw and reconstituted milk respectively. All the milk samples irrespective of types of milk were adulterated with cane sugar. Powder milk and added water were detected in all the raw and pasteurized milk samples besides starch was detected in all raw milk samples. Most alarming fact is hydrogen-per-oxide was detected in all of the raw milk samples though the pasteurized and reconstituted milk samples were free from any kind of preservative. The quality of reconstituted milk from the nutritional, physical and chemical aspects was good compared to raw and pasteurized milk but significant ($P<0.01$) variation were found among the hospitals. The TVC (Total Viable Bacterial Count) of all milk samples were found to be higher than acceptable limit. The highest TVC count was 1.50×10^6 CFU/ml in raw milk samples and the lowest was 1.25×10^4 CFU/ml in case of reconstituted milk samples. The highest TCC (Total Coliform Count) was 1.30×10^6 CFU/ml in raw milk samples and the lowest was 1.2×10^2 CFU/ml in reconstituted milk samples. On the other hand, *E. coli* was found negative in all of the samples but *S. aureus* was present in all of the milk samples. Among the *S. aureus* positive isolates, 50% were coagulase positive that indicates higher pathogenicity of the bacteria. This study indicates that all the milk samples collected from different hospitals were substandard and unsafe as far as microbial quality is concerned.

Keywords: Milk, physicochemical, nutritional, microbial quality, hospital.

CHAPTER – I

Introduction

Chapter I: Introduction

As an essential part of our daily diet, milk plays a vital role to meet up the increasing nutritional demand in developing countries like Bangladesh. Containing several nutrients, milk includes a number of vital constituents like protein, carbohydrate, fat, vitamins and minerals that make it an absolute food to consume (Komorowski and Early, 1992). Milk is a complex colloidal suspension containing fat globule, casein micelle and whey proteins in an aqueous solution of lactose, minerals and a few other minor compounds (Bhatia *et al*., 2015).

Usually, milk consumption is precisely related to the quality of milk which is availed from different sources (Kader *et al*., 2015). Physical properties of milk affect the composition and processing of milk (Bhatia *et al*., 2015). Physicochemical analysis is the first and foremost tool to monitor the quality of milk and milk products. Assessment of physicochemical properties is used to determine the concentration of milk components and to analyze the quality of milk products (Bhatia *et al*., 2015). Now a day, common people are concerned about the quality and safety of milk. According to World Health Organization (WHO) standards, quality milk should contain 3.5% protein, 2.6% fat, 7.71% solids-not-fat (SNF), 0.17% Titrable Acidity (TA), Specific Gravity of 1.030 and TVC of 1.3×10^6 CFU per ml (Hossain and Dev, 2013).The standards of milk and dairy products based on Bangladesh Standards (BDS) are mainly enrolled by Public health authority (BDS, 1985).

Due to rapid increase of population in Bangladesh, the demand of milk is growing faster (Kader *et al*., 2015). That is why; milk adulteration is a common phenomenon in Bangladesh. Adulteration of milk can lead to contamination and putrefaction of milk and milk products (Hossain and Dev, 2013). On the other hand, some fraudulent milk traders add non-food grade preservatives that are detrimental to health e.g. formaldehyde; that can leads to serious health hazards like cancer in a long run. So, detection of preservatives in milk is also an important task.

Though quality and safety are the most important issues but not easily maintained in less developing countries especially in hot tropics (DeGraaf *et al*., 1997). The fluid or semi-fluid nature of milk and its chemical composition renders it as one of the most supreme culture media for microbes (Mogessie and Fekadu, 1994).

At different stages of processing of milk, microorganisms may contaminate milk. Use of non-potable water is the most common pathway of germs into milk (Worku *et al*., 2012). Food borne illnesses is a major concern for the safety of milk and dairy products around the world. In developing countries, production of milk and various dairy products takes place under relatively unhygienic conditions and poor production practices, particularly in Bangladesh (Zelalem and Faye, 2006). The degree of microbial load in milk also depends on collection, sanitation, storage temperature and other factors related to milk handling and processing.

In recent years, the quality and safety of food are the absolute demands of the consumers of all over the world. A significant proportion of consumers get admitted into the hospitals as patient each year for treatment across the world, where milk is one of the common dietary ingredients provided by the hospital authority. Milk takes place into the diet of patients of all ages from the very ancient period due to its higher nutritious value and digestibility. So the assurance of quality and safety of milk that supplied to the patients of hospitals is also a vital task. For this study, different govt. and private hospitals of Chattogram Metropolitan Area (CMA) were selected. Total no. of existing hospitals in CMA is one medical college hospital, one general hospital and one hundred forty one (141) private clinics/ hospitals (DGHS, 2016). However, there is no report addressing this serious issue in our country till today. So considering the above view, present study was conducted with the following objectives.

Objectives

a) To analyze the nutritive value of the milk supplied in different hospitals of Chittagong Metropolitan Area (CMA).

b) To determine the microbial quality of milk supplied in different hospitals of CMA.

c) To determine the presence of certain pathogenic microorganisms (*E. coli* and *S. aureus*) in the supplied milk.

d) To make sure about the adulteration and added preservatives status in the supplied milk.

CHAPTER – II

Review of Literature

Chapter II: Review of Literature

2.1 Background

Milk is considered as a nearly complete food since it is a good source of protein, fat and major minerals. Also, milk and milk products are main constituents of the daily diet, especially for vulnerable groups such as infants school age children and old age (Davies *et al*., 1986). Fresh milk is defined as the whole, fresh, clean, lacteal secretion obtained by complete milking of one or more healthy animals excluding that obtained within fifteen days before or five days after calving or such periods as may be necessary to render milk practically colostrum free and containing the minimum prescribed percentage of milk fat (3.5%) and solids-not-fat (8.5%) (Goff and Hill, 1993).

2.2 Origin of milk

The term "milk" comes from "Old English ″meoluc″ (West Saxon) or ″milc″ (Anglian). The term milk has been defined under Codex Alimentarius Standards as: "the normal mammary secretion of milking animals obtained from one or more milking without either addition to it or extraction from it, intended for consumption as liquid milk or for further processing (Codex Alimentarius Commission, 2017).

2.3 A brief history of milk

At first, humans obscured to digest the milk of other mammals usually following the domestication of animals in the time of the Neolithic Revolution 9000–7000 BC in Mesopotamia to 3500–3000 BC in the Americas (Bellwood, 2005). At the outset, animals were conserved for meat, and archaeologist Andrew Sherratt (1981) has recommended that dairying, on the edge of the utilization of domestic animals for hair and labor, set about to a great extent following the additional products revolution in the fourth millennium BC. In the other part of the world, especially in the East and Southeast Asia, the Americas and Australia, milk and milk products were actually not great parts of the diet, conversely since they existed occupied by hunter-gatherers authority who did not hold animals or the rural agricultural economies did not insert domesticated dairy species. In the last 50 years, utilization of milk begins to be familiar equally. In the middle ages, milk was called the "virtuous white liquor" because alcoholic beverages were safer to consume than water.

2.4 Importance of milk

Milk is a complete nutritious food which contains essential nutrients including proteins, fat, and carbohydrates, vitamins and minerals, in the palatable forms (Kordylas, 1991). In many countries, milk and dairy products have become plays a vital part of the human diet over many years; thus significant recognition has been supported to upgrade the dairy production yield (Harding, 1999). Milk and dairy products contains variety of proteins as the whey proteins constitute about 18 % of the protein content of the milk and casein, a protein, which is found only in milk and approximately, 82 % of total proteins in milk is used as a standard for estimating proteins of other food as it contains all essential amino acids (Jensen, 1995). Furthermore, he added that protein is essential for building and repairing body tissues and antibodies which circulates in the blood and aids to resist infection. The most important nutrients of milk are proteins, calcium, potassium, phosphorus, vitamin A, riboflavin and thiamin (Kon, 1972). Milk is a comparatively low caloric food thus it is an enormous source of energy, besides high fat in milk of lower region breeds is a major part in people's diet (Payne, 1990).In addition, he also suggested that milk fat is absolutely digested and is essential for calcium absorption. Calcium, a mineral, found in milk which is easily absorbed by the human body, therefore, phosphorus plays a major role in calcium absorption and utilization, phosphorus is required in the proper ratio with calcium to form bone (Walstra, 2002). He also mentioned that milk is also a vital source of riboflavin (vitamin B_2), which helps to promote healthy skin and eyes, as well as vitamins A and D.

2.5 Components of milk

Milk is basically an oil-in-water emulsion which is composed of dissolved carbohydrates and protein compound with minerals. For the reason, it brings out as a major food item for the adolescent; almost all of its ingredients give strength for better health. The major specifications are energy (fat, lactose, and protein), biosynthesis ofnon-essential amino acids provided by proteins (essential amino acids and amino groups), essential fatty acids, vitamins and inorganic compounds, and water.

However, 97–98% triacylglycerol are present in milk fat & di- and mono acylglycerol, free cholesterol and cholesterol esters, free fatty acids, and phospholipids are also present in small amounts (Fox, 1995). The portion of milk fat contains fat soluble vitamins A, D, E, and K along with essential fatty acids such as linoleic and linolenic acid (McGee and Harold, 2004). The primary groups of milk protein are the caseins which are necessary for growth and development for nursing of the young's (Jensen, 1995). The major whey proteins in cow's milk are ß-lactoglobulin, which acts as an important protein in the synthesis of lactose and its presence is central to the process of milk synthesis (Walstra, 2002). Casein micelles are the greatest portion in the liquid part of milk. Four different types of casein proteins are: αs1-, αs2-, β-, and κ-caseins (Goff and Douglas, 2010). The vital carbohydrate in milk is lactose, approximately 59% of the solids-not-fat content of whole milk and about 30% of its calories (Filer and Reynolds, 1997). Milk accounts for 4.9% carbohydrate that is predominately lactose with trace amounts of monosaccharide and oligosaccharides (Holsinger, 1988). Major components of milk are carbohydrate, protein and fat used in the body with the help of vitamins. Milk is an essential source of thiamin, riboflavin and vitamins B_{12}. Milk contains a few amounts of niacin, pantothenic acid, vitamin B, vitamin C, and folate and is not considered as a vital source of these vitamins in the diet. Milk also contains the fat soluble vitamins A, D, E and K (Oste *et al*., 1997) Minerals play a significant role in the body such as enzymes functions, bone formation, water balance maintenance, and oxygen transport. Milk is an excellent source of calcium, magnesium, phosphorus, potassium, selenium, and zinc. Milk contains a few amounts of copper, iron, manganese, and sodium which are not considered as a major source of these minerals in the diet (Flynn *et al*., 1997)

2.6 Physicochemical properties of milk

2.6.1 Milk fat

From the beginning of lactation up to 10 weeks, the fat percentage reduced slowly and after that the fat percent increased getting as far as the maximum value 5.3% at the end of lactation period (Elbarbery *et al*., 1983). Furthermore, higher value of fat in milk gives its characteristic smoothness, flavor and color (Payne, 1990).

The minimum fat content is 3.0% and the maximum was 5.6% in cow's milk (Mahassin *et al*., 1988). Contrary, carbohydrates, protein and fat constituents in milk differs extensively in the formation caused by variation in genetics, nutritional factors and locational changes in various species (Fox, 1995).

2.6.2 Protein

Milk contains 3.3 % total protein and milk protein contains all 9 (Nine) essential amino acids required by humans. Total milk protein content and amino acid composition vary with cow breed and individual animal genetics. In cow's milk around 82% of milk protein is casein and the remaining 18% is serum or whey protein (Whitney, 1988)

2.6.3 Total solids (TS)

The total solids content of cow's milk in a dairy herd differed moderately from one season of the year to the other varying from 13.72 to 14.83 % (Khalifa *et al*., 1966). In different circumstances, higher value of totals solids content of cow's milk ranging from 12.13% to15.39 % (Khalid and Joseph, 1976).

2.6.4 Water

Cow milk constitutes about 87% water, as the delivery of milk from the dairy farm to the processing plant. The percentage of water ranges from 84.0 - 89.0 % even though, sometimes, an individual sample of authentic milk may exceed these ranges (walstra, 2002). The water percentage is also affected by any variation in the amount of other constituents (Eckles *et al*., 1951).

2.6.5 Acidity

The breakdown of lactose to lactic acid and other acids was responsible for the increase in the range of acidity (Gould, 1945). The titrable acidity of vendor's milk varies between 0.18 - 0.20 % as lactic acid (Ibrahim, 1973).

2.7 Food adulteration

Food adulteration is a global concern and developing countries are at higher risk associated with it due to lack of monitoring and policies. However, this is one of the most common phenomena that have been overlooked in many countries.

Unfortunately, in contrast to common belief, milk adulterants can pose serious health hazards leading to fatal diseases (Azad and Ahmed, 2016). Milk and dairy product adulteration came into global concern after breakthrough of melamine contamination in Chinese infant milk products in 2008 (Xin & Stone, 2008). However, history of milk adulteration is very old.

Possible reasons behind it may include- demand and supply gap, perishable nature of milk, low purchasing capability of customer and lack of suitable detection tests (Kamthania *et al*., 2014). The motivation for food fraud is economic, but the impact is a real public health concern (Ellis *et al*., 2012; Singh & Gandhi, 2015). The situation is significantly worse in developing and underdeveloped countries due to the absence of adequate monitoring and lack of proper law enforcement. Qualitative detection of adulterants in milk can be easily performed with chemical reactions. Nowadays, milk is being adulterated in more sophisticated ways that demands for cutting edge research for the detection of the adulterants (Azad and Ahmed, 2016).

2.7.1 Typical adulterants in milk

Adulterants in milk usually comprise addition of vegetable protein, incorporation of whey and watering which are known as economically motivated adulteration (Fischer *et al*., 2011; Singh & Gandhi, 2015). These adulterants do not lead to any serious health risk. But, some adulterants are too hazardous to be overlooked. Most of the major adulterants in milk having detrimental health effect are urea, formalin, detergents, ammonium sulfate, boric acid, caustic soda, benzoic acid, salicylic acid, hydrogen peroxide, sugars and melamine (Azad and Ahmed, 2016).

Instead of, the addition of water into milk to extend bulkiness, solidifying agents such as starch, flour, skimmed milk powder, whey powder or other ingredients to reverse the suspension and increase the hard contents of the milk (Fakhar *et al*., 2006); vegetable oil, sugarcane or urea to counteract the fat, carbohydrate or protein content of diluted milk. To extend the storage time of milk, various types of chemicals are used such as hydrogen peroxide, carbonates, bicarbonates, antibiotics, caustic soda and even the most fatal chemical formalin (Tariq, 2011).

Use of ice to increase the longevity of milk, to improve the superficial creation of milk by using detergents that reduces whipping properties and increases darkening of milk, to enhance white color of milk by turning over it an actual quality, calcium thioglycolate or potassium thioglycolate or calcium salts of thioglycolic acid is used (Walker *et al*., 2004). Formalin, salicylic acid, benzoic acid and hydrogen peroxide act as preservatives which increases the shelf life of the milk (Singh & Gandhi, 2015).

2.8 Food preservation

Raw milk is a perishable food item. Preservation of this nourishing drink is a great problem for marketing and for milk trader. Subsequently, procurement of raw milk from rural area to urban area, most of the time the milk traders fails to enhance the keeping quality of milk. Some local techniques are used to preserve raw milk throughout the time of transportation by putting water hyacinth leaves and date leaves etc. in milk. However, this technique degrades the quality of milk in an unhygienic condition and gives unsatisfactory results. Furthermore, this technique conducts the alterations of quality of raw milk which is unfit for consumption. As a consequence, milk is deteriorated to a great extent at the time of transportation. In the meanwhile, it is remarked that raw milk is deteriorated within 2 or 3 days in spite of storing in refrigerator. As a result of these facts, actions have been taken to establish preservation method for raw milk to prolong its storage time (Rokhsana *et al*., 2008)

Preservatives, a chemical substance, used to preserve food by inhibiting the growth of microorganisms and posterior spoilage occurring by fungus, mold, and rope inhibitors (Vollhardt *et al*., 1998). In addition, preservation by using chemicals in milk works as well as control for microbial toxins or by lowering the pH level of acidity which inhibits the growth of microorganisms (Australian Academy of Science, 2004). Preservation of milk by using hydrogen peroxide is an essential and cheap method for extending the keeping quality of milk throughout the time of transportation to the processing plants or market in farms in tropical developing countries (Odoi, 2003).Venden Berg (1985) has stated that preservatives may be used to improve the keeping quality of milk. The most common preservative is hydrogen peroxide.

2.8.1 Common preservatives in milk

2.8.1.1 Formalin

Formaldehyde, an aqueous solution of 37- 40% conc. is commercially available known as formalin. The usage of formalin for edible purposes is strictly prohibited because it has carcinogenic effect and inhibits the growth of all microorganisms including spores (Upadhyay *et al*., 2014).

2.8.1.1.1 Effect of formalin on physicochemical properties of milk

Many researchers have recommended that addition of formalin to milk increases the acidity quickly which holds throughout the time of storage. This certain increase in acidity has been caused by formalin-amino reactions. Dawood *et al*., (1974) observed that the increased titrable acidity ranges from 0.175 to 0.190% by the addition of 0.1% formalin to milk. Fahim *et al*., (1982) recommended that by the addition of 5ppm formalin to milk, increase the level of acidity percentage to 0.05%. Bansal and Singhal (1991) reported that after 2 months of storage, the buffalo milk samples have increased its viscosity with different levels of formalin and turned out it with viscous nature so that it is impossible to determine their viscosity by Ostwald viscometer. Many researchers have observed that milk samples which are preserved with formalin showed low fat value analyzed by Gerber method. Sharma and Sarwar (2000) found that by using Gerber method, fat contents of milk samples with formalin could not be analyzed properly. Although, Karmakar and Ghatak (1997) have observed that addition of 0.4% and 0.6% formaldehyde into milk samples preserved for up to 1 year, no significant changes in Gerber fat values. Bajaj and Rai (1992) reported that formaldehyde adversely affected the protein content determined by the Lowery method. However, formalin caused an immediate decrease in protein content of cow and buffalo milk by the dye-binding method. Formaldehyde is known to have an effect on fat and protein estimation. However, lactose content did not change, either on addition of formalin or during subsequent storage for up to six months at 30±2°C (Upadhyay *et al*., 2014).

2.8.1.1.2 Effect of formalin on microbiological quality of milk

Formalin interacts with the amino groups of adenine, cytosine, and guanine in the nucleic acid component, denaturing them and resulting in the inhibition of the growth of microorganisms (Haselkorn *et al*., 1961). Milk samples preserved with 0.4% formalin for 6 months did not show any mold growth (Bector *et al*., 1973). Bansal and Singhal (1991) showed that formalin-preserved cow and buffalo milk samples were almost free of bacterial growth for one year.

2.8.1.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is the traditional preservative to inhibit microbial proliferation and milk spoilage (Upadhyay *et al*., 2014). In milk, hydrogen peroxide may be utilized as a beneficial bactericide and has been recommended that as a method of enhancing milk quality in developing countries (Grindrod and Nickorson, 1967). Hydrogen peroxide may be used to increase the keeping quality of milk in certain cases (Rokhsana *et al*., 2008). Most of the H_2O_2 added is decomposed by the catalase-positive microorganisms. Its decomposition yields water and oxygen (Upadhyay *et al*., 2014).

2.8.1.2.1 Effect of H_2O_2 on physicochemical properties of milk

Giolitti (1949) found no changes for lactose, fat, total nitrogen and pH after the addition of 0.04% by weight of H_2O_2 to milk. According to other experiments, the lactose content of peroxide- treated milk was somewhat lower than that of untreated sample. H_2O_2 in higher concentration oxidizes proteins resulting in formation of aldehydes, ketones and acids, which are known sources of increased acidity (Antamer, 1993). H_2O_2 treatment (0.04% by weight) of milk increases the albumin content and decreases the casein content (Giolitti, 1949).

2.8.1.2.2 Toxic aspect of H_2O_2 in dairy

The effect of H_2O_2 treatment on preservation of the constituents of milk is less marked than that of other accepted processes applied in the dairy industry. The milk does not lose in nutrition value, apart from a small decrease of some vitamins and a considerable loss of ascorbic acid (Upadhyay *et al*., 2014).

2.9 Health effects of adulterants and preservatives

Peroxides and detergents, the two major adulterants in milk can lead to gastro-intestinal complications, which can cause gastritis and inflammation of the intestine. Due to the effects of undigested starch in colon, excessive starch in milk may lead to diarrhea. Since, accumulation of starch in the body may cause harmful for diabetic patients. Furthermore, carbonate and bicarbonates may lead to disruption in hormone signaling which regulate development and reproduction (Singuluri & Sukumaran, 2014). At concentrations above 0.1 mg/kg in air, inhaled formaldehyde can irritate the eyes and mucous membranes, resulting in watery eyes, headache, a burning sensation in the throat, and difficulty breathing. Large formaldehyde exposures, for example from drinking formaldehyde, solutions are potentially lethal. Formaldehyde is converted to formic acid in the body, leading to arise in blood acidity, rapid, shallow breathing, hypothermia, and coma or death people who have ingested formaldehyde require immediate medical attention. The potential health effects of sodium bicarbonate is inhalation, high concentrations of dust may cause coughing and sneezing (Tianig, 2009).

2.10 Microbiology of milk

Milk is globally consumed as nutritious food and it acts as a good territory for the proliferation of microorganisms such balanced diet. Several types of microorganisms contaminated milk which comes from the soil, water, or from the milk maid and/or skin and hair of the animals or utensils. Microbial contamination is caused by bacteria, virus and parasites (Champagne *et al*., 1993). Usually, microorganisms are transported to the food from natural sources for food contamination. Contamination starting from the point of handling and persists until consumption of food (IDF, 1976). Consequently, the contamination of these food products with the pathogenic microorganisms and its existence, development, proliferation and/ or causing infection is a major concern for public health. Some pathogens has been caused food-borne illnesses and among them *Staphylococcus spp*., *Escherichia coli* and *Salmonella spp*. tops the list (Haque *et al*., 2018).

Most of the pathogens present in milk are *coli*forms and psychotropic group of microorganisms (Cousin, 1982). Psychotropic groups of microorganisms include the gram positive genera (Bacillus, Clostridium) and the gram negative genera (*E. coli*, serratia), psychotropic bacteria are becoming dangerous to a great extent to the dairy industries because they produce heat resistant lipases and proteases outside of the cell (Cempirikova, 2002).

Milk-borne and milk-product borne outbreaks stand for 2-6% of bacterial food- borne outbreaks described by surveillance systems from several countries (Boor and Murphy, 2002). Milk, itself performs as a perfect media for microbial growth and development because of its most biodegradable nature (Hasan and Rakib, 2017). For concerning public health, ascertainment of microbial load is a major tramp in our country. Different types of techniques are established for the identification of microorganisms.in current study, Total viable bacterial count (TVC), Coliform count and *Staphylococcal spp.* were conducted as per recommendation of American Public Health Association (APHA, 1960). *Staphylococcal spp.* was conducted as per recommendation of American Public Health Association (APHA, 1960).

2.10.1 Total viable bacterial count (TVC)

Total bacterial count is a rough gauge to measure the quality of milk, herd health, efficacy of farm sanitation, milk handling and storage and transportation temperature. Total viable count (TVC), gives a quantitative estimate of the concentration of microorganisms such as bacteria, yeast or mold spores in a sample. A high TVC count indicates a high concentration of micro-organisms which may indicate poor quality for drinking water or foodstuff (Biyani *et al*., 2018). FDA (1997) reports showed that the total bacterial count of raw milk form Individual produces should not exceed milk 3000,000 CFU/ml while for pasteurized milk the bacterial load should not exceed 20,000 CFU/ml. Mohamed (1988) examined 240 samples of vendors milk for total bacterial counts and found that 54.4% had total bacterial counts with range between from 5.0×10^5 to 5.0×10^8 CFU/ml.

2.10.2 *Escherichia coli*

Due to its complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms. Among all micro-organisms, *Escherichia coli* is frequently contaminating organism, and is reliable indicator of fecal pollution generally in unsanitary conditions of water, food, milk and other dairy products (Diliello,1982). Martin *et al*., (1986) reported two cases of hemolytic uremic syndrome which provide evidence that raw milk may be a vehicle of transmission of *E. coli* O157: H7, both affected person consumed raw milk. Recovery of *E. coli* from food is an indicative of possible presence of enteropathogenic and/or toxigenic micro-organism which could constitute a public health hazard. Enteropathogenic *E. coli* (EEC) can cause severe diarrhoea and vomiting in infants and young children (Kumar *et al*., 2011).

The presence of Coliforms in food of animal origin indicates environmental and fecal contamination since these micro-organisms are abundant in the environment food (Shojaei and Yadollahi, 2008). *E. coli* is important as mastitis pathogens and widely distributed in the farm environment (Hogan and Smith, 2003). Amongst the coliforms, *Escherichia coli* organisms are the most common contaminants of raw and processed milk (Quinn *et al*., 2002). It is a reliable indicator of fecal contamination of water and food such as milk and dairy products (Todar, 2008).

2.10.3 *Staphylococcus aureus*

Milk and its derivatives are considered as a major source of *Staphylococcus aureus* infection in man (Zecconi and Piccinini, 1998).

In Europe, milk and other dairy products are found responsible for 5% of the staphylococcal outbreaks (Bianchi *et al*., 2014). *S. aureus* causes a variety of diseases in human and animals; the infections vary greatly in severity. There may be a mild skin infection to severe pneumonia and septicemia (Lowy *et al*., 1998). *S. aureus* can get access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling of raw milk (Scherrer *et al*., 2004; Jorgensen *et al*., 2005).

When the udder is infected, *S. aureus* may be excreted through milk in variable numbers up to 10^8 CFU/ml (Asperger and Zangeri, 2003). *S. aureus* may be pathogenic or non-pathogenic and the pathogenic strains are usually coagulase-positive and cause disease in their hosts. The infection may manifest as abscesses or mastitis to a severe toxic shock syndrome (Jahan *et al*., 2015). The bacteria may contaminate the milk during milking and the contamination depends on the sanitary condition of the plant, milking utensils and milking personals. Contamination may also result from the micro-organisms entering the udder through teat canal (Smith *et al*., 2007).

Staphylococcus aureus is an important foodborne pathogen, found in a good extent in milk as it is a nutrient enriched medium for their rapid growth and because of that milk and dairy products get frequently contaminated by this pathogen. Several researchers have reported the high prevalence of *Staphylococcus aureus* in the raw milk sample even in pasteurized milk as they can produce heat-stable enterotoxin (Loir *et al*., 2003; Zinke *et al*., 2012; Shanehbandi *et al*., 2014).

2.10.4 Procedures for screening of bacteria in milk

2.10.4.1 Direct microscopy

Initially, rapid assessment of milk samples is typically achieved using conventional microscopy, by which *Escherichia coli* appear as rod shaped, Gram-negative bacilli and *Staphylococcus aureus* as round shaped, Gram-positive cocci with grapes like clusters (Iorio *et al*., 2007).

2.10.4.2 Cultural properties

Blood agar

On bovine or ovine blood agar, *E. coli* appears as non-hemolytic, grey white moist, glistening, opaque, circular, convex colonies with entire edge (Soomro *et al*., 2002). Whereas *S. aureus* appears as glistening, smooth, entire, raised, translucent colonies that often have a golden pigment. The colonies are in 2-3 mm in diameter after 24 hrs. incubation and most strains show ß- haemolytic colonies (Bottone *et al*., 1984).

MacConkey agar

MacConkey agar is an indicator, a selective and differential culture medium for bacteria designed to selectively isolate Gram- negative and enteric (normally found in the intestinal tract) bacilli and differentiate them based on lactose fermentation**.** The crystal violet and bile salts inhibit the growth of Gram-positive organisms which allows for the selection and isolation of gram-negative bacteria. Enteric bacteria that have the ability to ferment lactose can be detected using the carbohydrate (lactose) and the pH indicator neutral red. *Escherichia coli* produce smooth, circular pink colonies with spreading growth (Anderson *et al*., 2013).

Mannitol salt agar

Mannitol salt agar or MSA is a commonly used as selective growth medium to isolate *S. aureus*. It encourages the growth of *S. aureus* while inhibiting the growth of others. This medium contains a high concentration (about 7.5–10%) of salt (NaCl), making it selective for *staphylococci* since this level of NaCl is inhibitory to most other bacteria. It is also a differential medium for mannitol-fermenting *staphylococci*, containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting *staphylococci* such as *Staphylococcus aureus* that produces yellow colonies with yellow zones, whereas other produce small pink or red colonies with *staphylococci* produce small pink or red colonies with no color change to the medium (Anderson *et al*., 2013).

2.10.4.3 Biochemical properties

2.10.4.3.1 Tests for *Escherichia coli*

Indole test

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to convert tryptophan into indole. This division is performed by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase."

Like many biochemical tests on bacteria, results of an indole test are indicated by a change in color following a reaction with an added reagent.

A positive result is shown by the presence of a red or red-violet color in the surface alcohol layer of the broth. A negative result appears yellow. A variable result can also occur, showing an orange color as a result. This is due to the presence of skatole, also known as methyl indole or methylated indole, another possible product of tryptophan degradation. *E. coli* showed positive result for cleaving indole (MacFaddin, 1980).

Triple Sugar Iron (TSI) test

The Triple Sugar Iron (TSI) test is a microbiological test roughly named for its ability to test a microorganism's ability to ferment sugars and to produce hydrogen sulfide. Bacteria that ferment any of the three sugars in the medium will produce byproducts. These byproducts are usually acids, which will change the color of the red pH-sensitive dye (phenol red) to a yellow color. Position of the color change distinguishes the acid production associated with glucose fermentation from the acidic byproducts of lactose or sucrose fermentation. Many bacteria that can ferment sugars in the anaerobic butt of the tube are enterobacteria. *E. coli* represents the TSI test by fermentation of lactose and production of acid and H_2S (Tille, 2014).

2.10.4.3.2 Tests for *Staphylococcus aureus*

Catalase test

S. aureus produce abundant catalase, which can interact with hydrogen peroxide to produce oxygen. The test distinguishes catalase-producing cocci (e.g., *staphylococci*) from non-producers (e.g., *streptococci*). Catalase test cannot be performed on blood agar because blood contains catalase (Davis and Hoyling, 1973).

Coagulase test

Coagulase produced by certain Gram-positive cocci, including *S. aureus* either in bound form (attached to the bacterial cell wall) or as free enzyme, converts fibrinogen to insoluble fibrin in the presence of plasma, resulting in clotting. Presence of coagulase distinguishes *S. aureus* from coagulase-negative *Staphylococci* (CoNS) (Brown *et al*., 2005).

2.11 Quality and safety issues of milk in hospitals of BD

Milk is the most diversified natural food in terms of composition; a rich source of major and minor components which is essential to provide the nutritional requirements for human body. Due to its high biological value, milk is an ideal food for all including patients suffering from different diseases. Many sick people go to hospitals for treatment around the world, where milk is provided as nourishing drink to the admitted patients for their faster recovery. If the milk supplied in the hospitals is not of standard quality, it will cause many food borne illnesses to the immune compromised patients.

Recently, quality and safety of milk has an alarming issue in all over the world as well as in Bangladesh. Due to its high perishable nature, different types of adulterants and preservatives are added into the milk to increase the commercial life that can leads to serious health hazards. Unhygienic milking condition, improper handling of milk, storage temperature etc. cause high microbial contamination. For this reason, analysis of chemical and microbial quality of milk which is supplied at different hospitals for patients is necessary. Till now there is no research published on this alarming issue in Bangladesh. Therefore, considering this view, the present study was conducted to know the overall quality of milk samples which are provided to patients by hospital authority at CMA.

CHAPTER – III

Materials and Methods

Chapter III: Materials and Methods

3.1 Study Area

A cross sectional study was carried out for assessing the quality of milk from different hospitals of Chattogram Metropolitan Area (CMA). The collected samples were tested in Dairy Science Laboratory for physicochemical properties and in Poultry Research and Training Centre (PRTC) for microbial quality at Chattogram Veterinary and Animal Sciences University (CVASU) during the period from 18th April to 15th December 2019.

Figure 1: A map of Chattogram showing the locations of sampling areas

3.2 Selection of hospitals

A total of nine (9) government and private hospitals located in CMA were selected for this study. The selected hospitals are Chattogram General Hospital (A), Chattogram Maa-O-Shishu Hospital (B), CSCR Hospital (C), Chattogram Diabetic General Hospital (D), Chattogram Eye Infirmary Hospital (E), Chattogram Metropolitan Hospital Ltd. (F), Medical Centre Hospital (G), Chattogram Medical College Hospital (H), Chattogram Railway Hospital (I) (Appendix, Figure 26-34).

3.2.1 Criteria for selecting the hospitals

From the study areas, following categories were considered for the selection of hospitals. These are

- Easy access to hospital
- Interest of hospitals
- Convenient distance
- Easy to handle of milk samples
- Good transportation facilities

3.3 Sampling

Samples were collected aseptically from the milk vessel to sterilized falcon tubes (Figure 2). After collection, the samples were kept into ice box around 1-2 hrs. for transporting (Figure 3) and then tested as soon as reached to the laboratory. The replication number of samples from each hospital was three.

Figure 2: Collection of milk sample by using sterile 50 ml felcon tube

Figure 3: Sample kept in ice box during transportation

3.4 Physicochemical Analysis

3.4.1 Physical Analysis

In case of physical characteristics of milk samples, only specific gravity was performed following the standard procedure as described by Indian Standards (1961). Specific gravity was determined by using quevenne lactometer.

3.4.1.1 Determination of specific gravity of milk

Initially, milk sample was mixed well. Sufficient milk was poured into the lactometer jar up to its brim to allow the lactometer to float freely. Then the lactometer was placed to the jar in rotating moment and allows it to a constant level. The reading was taken at stationary phase (Figure 4). The temperature of milk sample was recorded with the help of dairy thermometer. Corrected Lactometer Reading (CLR) was calculated by adding 0.2°F for each above 84°F or deducting 0.2°F for each below 84°F.

Figure 4: Determination of specific gravity by using quevenne lactometer at stationary phase

Calculation

Corrected Lactometer Reading (CLR)

At first, lactometer reading was determined. Then temperature of the supplied milk sample was recorded.

Calculation for Specific Gravity

$$\text{Specific Gravity} = \frac{\text{CLR}}{1000} + 1$$

We know, Corrected Lactometer Reading (CLR),

$$\text{CLR} = \text{LR} \pm (\Delta°\text{F} \times 0.2)$$

3.4.2 Chemical Analysis

Different chemical properties of milk has been determined such as fat%, total solids (TS)%, solids-not-fat (SNF)% following the standard procedures as described by FAO (1984). The protein percentage was determined by following the method of Payne (1932). Titrable acidity (TA) was determined by titrating with 0.1N sodium hydroxide solution following the method of Aggarwala and Sharma (1961).

3.4.2.1 Determination of acidity percentage of milk

At first, milk sample was mixed well and 10 ml milk sample was taken in a 100 ml porcelain beaker. Then 3 to 4 drops of phenolphthalein indicator was added to the sample. At last, the mixture was titrated by using standard 0.1N NaOH solution up to the appearance of faint pink color (Figure 5). The same procedure was repeated for 3 times. The volume of alkali used for each titration was recorded. Acidity of milk was measured in terms of % of lactic acid using the following formula.

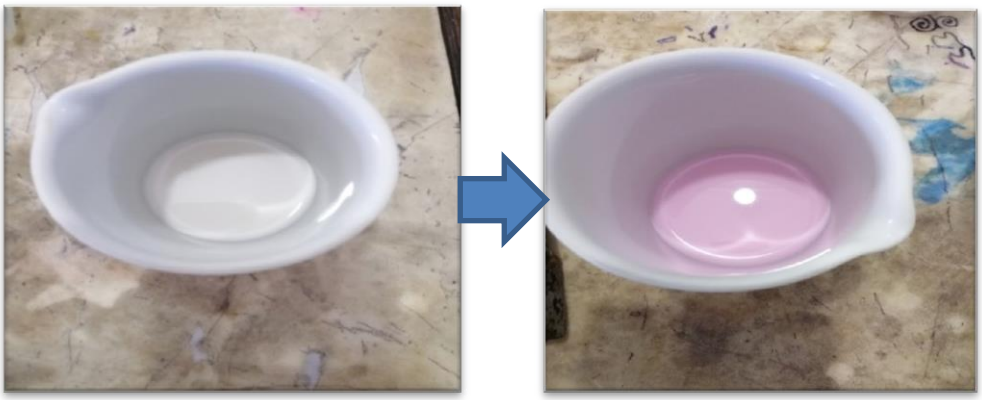

Figure 5: Appearance of faint pink color indicating end point of titration

Calculation

$$\% \text{ of lactic acid} = \frac{\text{Volume of alkali used} \times (\text{N}) \text{of NaOH} \times 0.09}{\text{Volume of milk sample}} \times 100$$

3.4.2.2 Determination of fat percentage of milk by Gerber Method

At first, 10 ml sulphuric acid was taken in a butyrometer. 10.75 ml well mixed milk sample was taken and added to it.1 ml amyl alcohol was also added to it. The opening was closed by the lock stopper and tightens the stopper. The contents were shaken well at 45° angles until the disappearance of white particle. Butyrometer was centrifuged for 5 minutes at 1100 rpm. Butyrometer was hold in vertical position. Then the fat column was adjusted within the scale on butyrometer and reading was recorded (Figure 6).

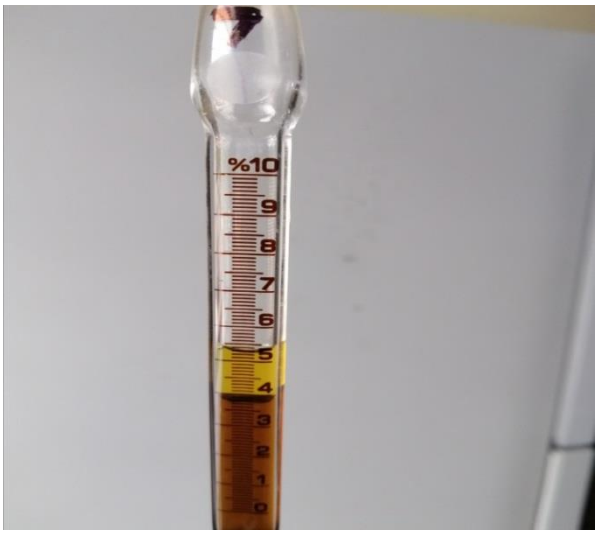

Figure 6: Reading of fat recorded from butyrometer at vertical position

3.4.2.3 Determination of protein percentage of milk

Firstly, 10 ml milk sample was taken in a conical flask. 0.4 ml potassium oxalate was added, mixed and kept rest the mixture for two minutes. After that 2-3 drops of phenolphthalein indicator was added to the mixture. Then the mixture was titrated against 0.1N NaOH solutions up to the appearance of faint pink color. 2 ml formaldehyde solution was added to the mixture and kept rest for 30 minutes. Again titration was done following the same procedure after adding 2-3 drops of phenolphthalein indicator. Amount of alkali required (TV) was recorded and the protein percentage was calculated.

Calculation

Protein Percentage = ml of alkali required × 1.70

3.4.2.4 Estimation of SNF% and Total Solids (TS) % of milk

The milk sample was mixed well. Fat % of milk was determined by Gerber method. The corrected lactometer reading was calculated at 84°F standards. The SNF% and TS% were estimated by the following formula.

Calculation

Indian Standard Institution Formula

$$\%SNF = \frac{CLR}{4} + 0.25 \times F\ (Fat\ \%) + 0.6$$

$$\%TS = \%Fat + \%SNF$$

3.5 Determination of adulterants in milk

The adulterants were tested by following the protocol of Food Safety and Standards Authority of India (2015).

3.5.1 Water adulteration test

It was done by using the lactometer reading of milk sample. At first, raw milk was poured into a 100 ml measuring cylinder. Then, a lactometer was dropped in the milk to slowly sink down. Further, the lactometer reading was taken (Figure 7) and recorded Lactometer degree (°L).

Figure 7: Lactometer reading
(Adulteration with water)

3.5.2 Starch test

Two milliliter well mixed milk was taken in a test tube. A few drops of 5% iodine solution were added. Change of color to blue indicated that the milk was adulterated with starch (Figure 8).

Figure 8: Presence of blue color indicated positive starch test

3.5.3 Cane-sugar test

Two ml well mixed milk sample was taken in a test tube. Then 1 ml of HCl was added in the same test tube. 0.1g resorcinol was also added there. Then the test test tube was shaken well and placed in a hot water bath at 60°C for 2-3 minutes. Appearance of red color precipitation indicated the presence of added sugar in milk (Figure 9).

Figure 9: Red color precipitation indicates positive cane sugar test

3.5.4 Powder milk test

At first, 10 ml well mixed milk sample was taken in a screw capped test tube. Then a few drops of formalin were added in that solution. Then the test tube was placed in the hot water bath at 60°C for 10 minutes. If peculiar odor comes out from the test tube, indicated the presence of powder milk.

3.6 Determination of added preservatives in milk

In milk, preservatives are used to increase the commercial life of milk but all preservatives are not compatible to the body. So, detection of non- food grade preservatives in milk were performed following the standard procedures as describedby Food Safety and Standards Authority of India (2015).

3.6.1 Formalin detection test (Hehner's Test)

Two milliliter well mixed milk sample was taken in a test tube. Then a drop of ferric chloride was added. Dilution was done three times of its volume with water. Conc. sulfuric acid was run along the side of the test tube. Appearance of a purple ring at the junction of the two layers indicated the presence of formalin and greenish color showed the absence of formalin (Figure 10).

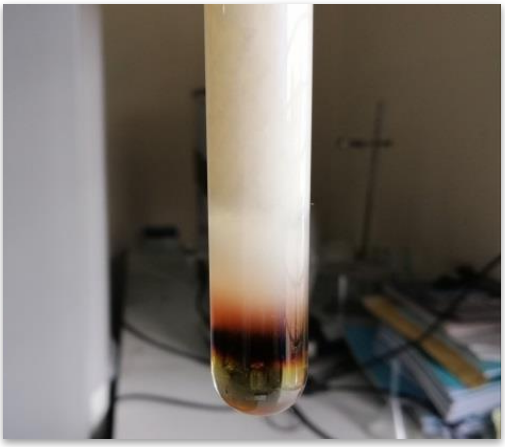

Figure 10: Negative result (no violet ring) of formalin test

3.6.2 H_2O_2 detection test

Five ml of milk sample was taken in a test tube. A drop of Paraphenyle-diamine-hydrochloride was added. If blue color developed in the tube indicated the presence of H_2O_2 in milk (Figure 11).

Figure 11: Bluish ash color indicates positive result of H_2O_2 test

3.6.3 Carbonate or bi-Carbonate detection test

Five ml of milk sample was taken in a test tube. A drop of resolic acid was added to it. If brownish yellow color developed in the tube indicated the absence of carbonate in milk (Figure 12). Appearance of red rose color indicated the presence of carbonates in milk.

Figure 12: Presence of brownish yellow color indicating negative Carbonate or Bi- Carbonate test

3.7 Bacteriological analysis

Total viable bacterial count (TVC), Total Coliform Count (TCC), isolation of *Escherichia coli* and *Staphylococcus aureus* were performed to determine the microbial quality of samples collected from different hospitals of CMA. Different bacteriological media such as Plate count agar (TVC), MacConkey agar (TCC) and Mannitol salt agar (*S. aureus*) were used (APHA, 1992).

Procedure

3.7.1 Preparation of agar medium

All media were prepared according to the manufacturers' directions (Figure 13). The medium was dispersed in different screw capped bottle & sterilized in autoclave at 121°C for 15 minutes. After sterilization the bottle were placed in water bath at 45°C until used. It is recommended that the temperature of hot water bath should not exceed 50°C.

Figure 13: Preparation of different agar medium (Macconkey, PCA & MSA)

3.7.2 Enumeration of total viable bacterial count (TVC)

3.7.2.1 Spread Plate Technique

1. At first a series of test tubes (5), each containing 9 ml diluents were taken.

2. Fifty (50) ml milk sample was homogenized in 450 ml diluents and making suspension in a beaker.
3. From the original sample, 1 ml was transferred in the test tube no.1 and mixed thoroughly.
4. 1 ml from 1st test tube to 2nd test tube was transferred and continues up to last one & 1 ml was discarded from the last test tube (Figure 14).
5. From each tube, 3 petri dishes were taken containing PCA media.
6. Then 0.5 ml mixture was transferred from each of the test tube to another tube. One pipette should be used for one tube.
7. Tips of the test tube should be touched gently to the media.
8. Diluted samples were spread over the surface of the media using glass spreader or sterilized swab stick.
9. The petri dishes were marked (sample no, date etc.) and kept in incubator in inverted position at 37°C for 24 to 72 hrs. to facilitate viable bacterial growth.
10. After 1 day interval up to 3 days after incubation the colonies were observed (Figure 15).
11. In which plate colony counted were 30-300 should be included and others should be discarded.
12. The colonies of three plates per samples were counted and made average to them (Figure 16).
13. Following counting, calculation of total count was expressed as colony forming units per milliliter (CFU/ml).

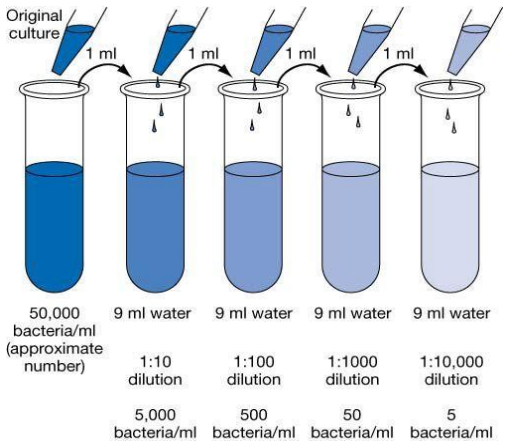

Figure 14: Serial dilution by using tenfold technique

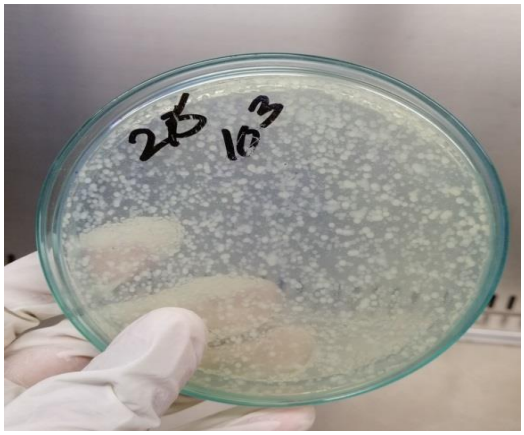

Figure 15: Total viable bacterial count (TVC)

Figure 16: Use of colony counter for TVC

Calculation

Total no. of bacteria = No. of colonies × Dilution factor

3.7.3 Enumeration of total coliform count (TCC)

TCC was determined by the same method used in the enumeration of total viable bacteria. Nutrient agar medium MacConkey agar was used for enumeration of total coliform count. For the determination of TCC, 1ml of each tenfold dilution was transferred to MacConkey agar plate. For each dilution, three petridishes were taken containing MacConkey agar. Diluted samples were inoculated on the surface of the media using glass spreader or sterilized swab stick .All the inoculated plates were incubated aerobically at 37°C temperature for 24 hrs. After 1 day interval incubation period, typical pinkish and centrally red colonies were considered as positive (+ve) for coliform (Figure 17). A single, isolated colony was picked up by using a sterile loop from the plates, and representative colonies were sub cultured again smearing on MacConkey agar plates to obtain pure cultures of isolates (Cheesbrough, 1985).

Figure 17: Pinkish & centrally red colored colonies observed on MacConkey agar plate (TCC)

3.7.3.1 Isolation of bacteria in pure culture

The mixed culture was inoculated into nutrient agar media by streaking into plate to obtain well isolated colonies for isolation of bacteria in pure culture. Firstly, by using a sterile inoculating loop, an inoculum was picked up and spread on an area of the medium in the petridish. The inoculating loop was sterilized by being heated as red hot in a flame. By drawing the cooled parallel line, the inoculum was spread over the plate (Figure 18). To obtain a culture containing only one type of colony, this method was repeated as many times as necessary and usually at least two more times to ensure pure isolates (Cheesbrough, 1985).

Figure 18: Streaking a well isolated colony for pure culture by using inoculating loop

3.7.3.2 Isolation of *E. coli*

Eosin methylene blue (EMB) agar was used for the confirmation test for *E. coli* (Figure 19). A loop full of suspension was streaked onto the plates from confirmed positive brilliant green bile broth culture. Inoculated plates were incubated at 35°C for 18 to 24 hrs. After incubation, discrete dark centered nucleated colonies with metallic sheen were observed which indicates as a positive result from each (EMB) agar plate. Two colonies or more were picked and cultured on nutrient agar slants for morphological examination of gram negative *E. coli* (Cheesbrough, 1985).

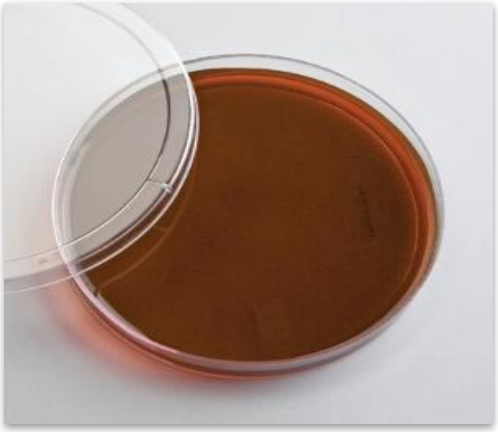

Figure 19: No discrete dark centered nucleated colonies with metallic sheen observed on EMB agar plate

3.7.3.3 Identification of *Escherichia coli*

3.7.3.3.1 Morphological characterization of organisms by Gram's staining method

At first, a glass slide was taken for gram's staining. A loop full of sterile distilled water was placed in the center of a sterile, clean, dry, grease free glass slide. On the slide, a single colony with similar characters was picked up with an inoculating loop and was mixed with distilled water. The colony was shaped to thin smear on the slide. The smears were fixed by drying into air. 0.5% crystal violet solution was then applied on the smear for 1 minute. Then Gram's iodine solution was added for 1 minute to act as mordant. Acetone alcohol was then used to decolorize for 1-2 seconds. Then the slide was rinsed with water. Safranin solution (2 %) was applied as a counter stain and allowed to stand for 1 minute. Then the slide was rinsed with water. Finally, the slide was blotted with blot paper and was allowed to air dry. The slide was examined under light microscope with high power objective (100X) using immersion oil (Merchant and Packer, 1969).

3.7.3.3.2 Biochemical characterization

All the positive samples were further confirmed using bio-chemical examinations and described below:

Indole test

Before performing the test, 5 ml of pure bacterial culture was inoculated with 2 ml of sterile tryptophan or peptone broth and incubated at 37°C for 24-48 hours.

Following incubation, 0.5 ml of Kovac's reagent (isoamyl alcohol, para-Dimethyl-amino-benzaldehyde, concentrated hydrochloric acid) was added to the culture broth and mixed thoroughly. Then the tube was allowed to stand for a while. The presence of a red or red-violet color in the surface alcohol layer of the broth indicated the positive result for *E. coli*. A negative result appears yellow (Cheesbrough, 1985).

Triple Sugar Iron Agar (TSI) test

Preparation of TSI agar medium

At first, combined the ingredients, and adjusted the pH to 7.3. Then boiled to dissolve the agar and dispensed into tubes. Then, sterilized by autoclaving at 121°C for 15 minutes and cooled in a slanted position to give a 2.5 cm butt and a 3.8 cm slant. TSI agar is also available commercially (Carter, 1986).

Procedure for Triple Sugar Iron Agar (TSI) test

With a sterilized platinum loop, a well-isolated colony was picked up. This colony inoculated into TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. Loosened the cap of the tube and incubated the tube at 35°C in ambient air for 18 to 24 hours. If lactose (or sucrose) was fermented, a large amount of acid was produced, which turned the phenol red indicator yellow both in butt and in the slant. It was indicated the presence of *E. coli* (Carter, 1986).

Methyl Red (MR) test

The Methyl Red (MR) test was conducted by inoculating a pure colony of the test organism in 5 ml sterile glucose phosphate peptone broth. After 48 hours incubation at 37°C, 5 drops of methyl red indicator was added. A red coloration was positive for the presence of *E. coli* and indicated an acid pH of 4.5 or less resulting from the fermentation of glucose (Cheesbrough, 1984).

3.7.4 Isolation of *S. aureus*

To screen *S. aureus* in the collected milk samples the following media were used:

i Selective media: Mannitol Salt agar (HiMedia®, India)

ii Enriched media: Blood agar (Oxoid Ltd.), Brain Heart Infusion Broth (Oxoid Ltd.),

Specific media, Mannitol Salt Agar (MSA) was used for the growth of *Staphylococcus aureus*. A loop full of milk sample were spread and streaked respectively onto the Mannitol Salt agar (HiMedia®, India) and then incubated aerobically at 37°C for 24-48 hrs. After incubation, the bacterial growth was observed.

The presumptive colonies of *S. aureus* were further cultured onto mannitol salt agar (MSA) and repeatedly sub-cultured to get pure culture. The isolates also fermented mannitol with the color change of MSA (Mannitol Salt Agar) and production of small yellow colonies (Figure 20).

Figure 20: Fermentation of mannitol salt agar by *S. aureus* producing small yellow colored colony

Any colonies yielding golden yellow color were suspected for *S. aureus* (Anderson *et al*., 2013), which were further sub- cultured onto blood agar (Figure 21). Initially, any smooth colonies on blood agar producing beta-hemolysis (Bottone *et al*., 1984) were considered for the growth of *S. aureus*, but confirmed by Gram's staining and a set of recommended biochemical tests, as described below:

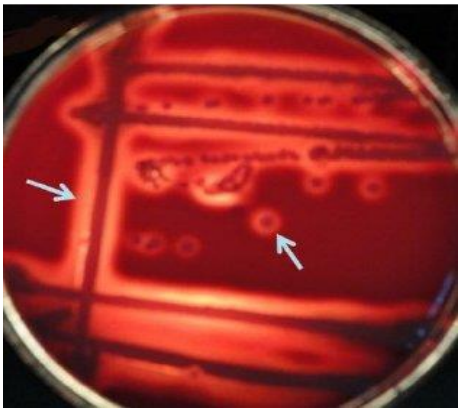

Figure 21: ß- hemolysis showed on blood agar

3.7.4.1 Identification of *S. aureus*

3.7.4.1.1 Gram's staining

Gram's staining was performed according to the conventional procedures. A loop full of sterile distilled water was placed in the center of a sterile, clean, dry, grease free glass slide. On the slide, a single colony with similar characters was picked up with an inoculating loop and was mixed with distilled water. Briefly, a portion of a suspected colony on a blood agar was thin-smeared over a slide, heat fixed, stained with crystal violet treated with Gram's iodine, decolorized with acetone-alcohol and finally counter stained with safranin. The slide was then microscopically examined. Gram-positive cocci with cluster like cellular arrangements (Figure 22) were suspected for the presence of *S. aureus* (Iorio *et al*., 2007).

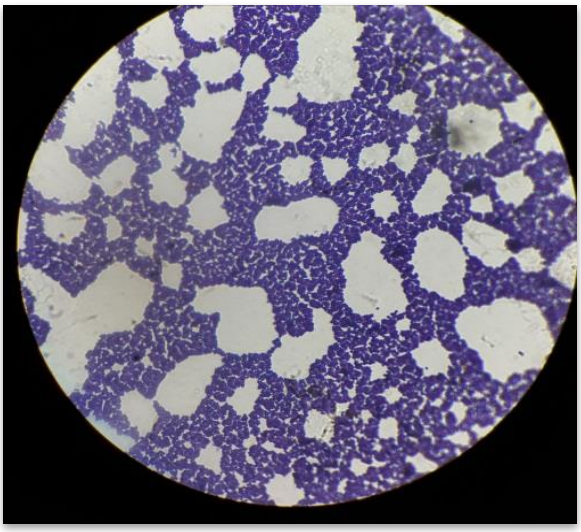

Figure 22: *S. aureus* with characteristic Gram- staining properties under the microscope (captured at 1000x)

3.7.4.1.2 Biochemical tests

Catalase test

A solution of 3% hydrogen peroxide ($H_2O_{2)}$ was used to accomplish the catalase test. Suspected colony was taken on clean, dry, grease free glass slide and mixed with loop full water and then one drop of 3 % H_2O_2 was added. Positive result was indicated on the basis of production of vigorous bubble formation (Figure 23). This test could also be done directly on suspected colony on agar media, but in case of blood agar, it could give false positive result (Davis and Hoyling, 1973).

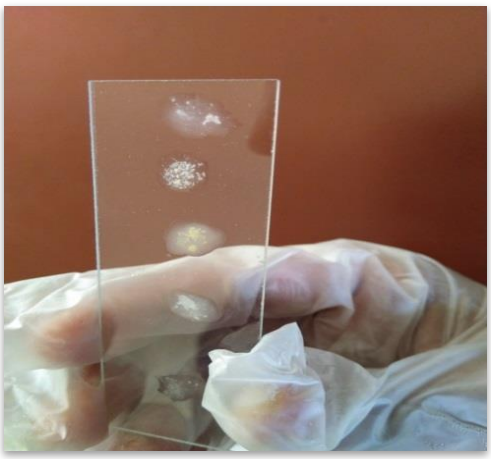

Figure 23: Formation of bubble in catalase test

Coagulase test

Pathogenic *Staphylococcus aureus* is known to produce coagulase, a virulence factor that can clot plasma into gel in tube or agglutinate cocci on slide. Most pathogenic strains of *S. aureus* produce two types of coagulase, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellularly, bound coagulase is a cell wall associated protein. Free coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test. Slide coagulase test may be used to screen isolates of *S. aureus* and tube coagulase test may be used for confirmation (Brown et al., 2005).

Collection of plasma

Due to the convenience and availability, coagulase test was accomplished using horse plasma. Horse blood was collected aseptically in a sterile blood collection bag with anticoagulant. Then the blood was transferred into sterile test tubes and centrifuged at 2600 rpm for 10 minutes. Then the plasma was accumulated as supernatant was carefully collected by sterile syringe and kept at -20°C for future use.

Slide coagulase test

Dense suspensions of *Staphylococci* from culture were made on two ends of a clean glass slide. On end was labeled as “test” and the other as “control”. The control suspension used to rule out false positivity due to auto agglutination. The test suspension was treated with a drop of citrated plasma and mixed well. Agglutination or clumping of cocci within 5-10 seconds was taken as positive (Brown et al., 2005).

Tube coagulase test

Three test tubes were taken and labeled "test", "negative control" and "positive control". Each tube was filled with 0.5 ml of 1 in 10 diluted horse plasma. To the tube labeled test 0.1 ml of overnight broth culture of a test isolate was added. To the tube labeled positive control 0.1 ml of overnight broth culture of known *S. aureus* was added and to the tube labeled negative control 0.1 ml of sterile broth was added. All the tubes were incubated at 37°C and observed up to 4 to 24 hours. A positive result was indicated by gelling of the plasma (Figure 24), which remained in place even after inverting the test tube (Ryan and Ray, 2004).

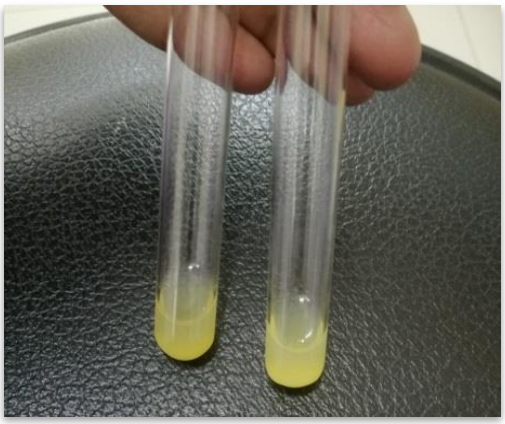

Figure 24: Formation of clotting in tube coagulase

3.7.4.1.3 Preservation of the isolates

All coagulase positive isolates were inoculated into Brain Heart Infusion Broth (BHI) from blood agar (Figure 25), incubated overnight at 37°C and then preserved at -80°C with 50% glycerol (v/v) in 1.5 ml eppendorf tubes (700µl broth culture and 300µl glycerol) for further investigation (Figure 26).

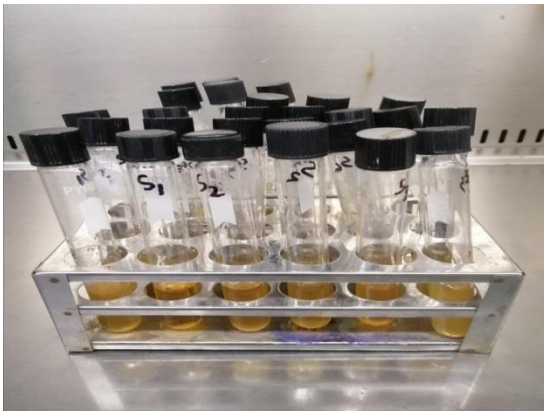

Figure 25: Inoculation of positive isolates into BHI broth

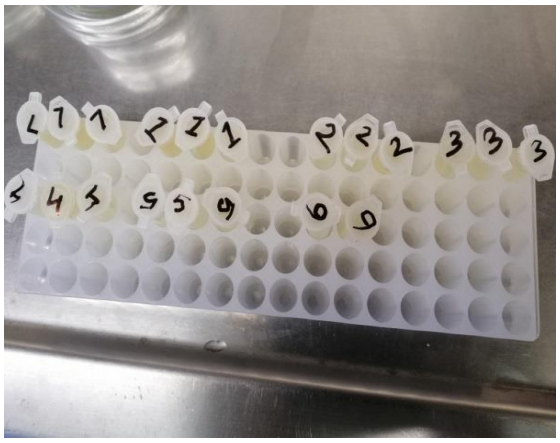

Figure 26: Preservation of isolates by 50% glycerol

3.8 Statistical Analysis

The data were recorded and categorized in Microsoft Office Excel 2010 spread sheet and finally analyzed by Compare means One-way ANOVA by using statistical software SPSS 2017 (14.2 version).

CHAPTER – IV

Results

Chapter IV: Results

Table 1 shows that the reconstituted milk was supplied by the five hospitals and both reconstituted and raw milk by one hospital to the patient. Pasteurized milk was supplied by only one hospital and raw milk by two hospitals (Appendix, Figure 35-47).

Table 1. Types of milk supplied by hospitals

Hospitals	Types of milk supplied to the patients
A	Raw milk
B	Reconstituted milk
C	Reconstituted milk
D	Raw milk
E	Reconstituted milk
F	Reconstituted milk
G	Reconstituted milk
H	Pasteurized milk
I	Raw milk, Reconstituted milk

[A= Chattogram General Hospital, B= Chattogram Maa – O- Shishu Hospital, C= CSCR Hospital, D= Chattogram Diabetic General Hospital, E= Chattogram Eye Infirmary Hospital, F= Chattogram Metropolitan Hospital Ltd., G= Medical Centre Hospital, H= Chattogram Medical College Hospital, I= Chattogram Railway Hospital]

4.1 Physicochemical properties of milk

Among the raw milk samples, lowest fat percentage 0.5±0.058 in case of hospital A and the highest was 2.2±0.058 in case of hospital I as shown in Table 2. In case of protein percentage, the highest percentage was 4.43±0.012 in hospital D and the lowest percentage was 3.5±0.058 in hospital I. Table 2 shows that the highest SNF percentage was 7.51±0.116 in hospital D and the lowest was 7.23±0.056 in hospital I. The highest TS percentage of raw milk was 9.5±0.058 in hospital I and the lowest was 8.0±0.115 in hospital A. The highest TA percentage of raw milk was 0.213±0.001 in hospital A and the lowest TA percentage was 0.137±0.002 in hospital I. Average specific gravity was lowest 1.025±0.0005 in case of hospital I and the highest in case of hospital A and D was 1.027±0.0005 shown in table 2. The average fat%, protein%, SNF%, TS%, TA% and specific gravity of raw milk was 1.1±0.281, 4.01±0.141, 7.41±0.068, 8.44±0.274, 0.18±0.011 and 1.026±0.0004 respectively. The mean difference of fat, protein, TS and TA percentage between hospitals in case of raw milk samples were statistically significant ($p<0.01$).

Table 2. Nutritional quality of raw milk supplied in different hospitals of CMA

Hospital IDs	Parameter (Mean±SE)					
	Fat%	**Protein%**	**SNF%**	**TS%**	**Titrable Acidity (TA)%**	**Specific Gravity**
A	0.5^{b}±0.058	4.1±0.115	7.5±0.115	8.0^{b}±0.115	0.213^{a}±0.001	1.027^{a}±0.0005
D	0.6±0.173	4.43^{a}±0.012	7.51^{a}±0.116	8.12±0.006	0.195±0.001	1.027^{a}±0.0005
I_1	2.2^{a}±0.058	3.5^{b}±0.058	7.23^{b}±0.056	9.5^{a}±0.058	0.137^{b}±0.002	1.025^{b}±0.0005
Average	1.1±0.281	4.01±0.141	7.41±0.068	8.44±0.274	0.18±0.011	1.026±0.0004
SEM	0.281	0.141	0.068	0.274	0.011	0.0004
p-value	0.001	0.001	0.156	0.001	0.001	0.078
Level of Significance	**	**	NS	**	**	NS
FAO Standards	3.5	3.3	8.7	12.2	-	-

[In the above table, A= Chattogram General Hospital, D= Chattogram Diabetic General Hospital, I= Chattogram Railway Hospital (I_1= Raw milk). a= Highest value, b= Lowest value, SE= Standard error, SEM= Standard error mean, **= statistically significant ($p<0.01$), NS= Not significant, FAO= Food and Agricultural Organization. Means with different superscript(s) in the same column differ significantly]

Table 3. Nutritional quality of pasteurized milk supplied in selected hospital of CMA

Hospital IDs	Parameter (Mean±SE)					
	Fat%	**Protein%**	**SNF%**	**TS%**	**Titrable Acidity (TA)%**	**Specific Gravity**
H	1.7±0.115	4.25±0.144	7.09±0.054	8.46±0.258	0.14±0.001	1.025±0.0012
Average	1.7±0.115	4.25±0.144	7.09±0.054	8.46±0.258	0.14±0.001	1.025±0.0012
SEM	0.115	0.144	0.054	0.258	0.001	0.0012
Bangladesh Standards	3.5	3.3	8.0	11.5	0.15	1.028-1.032

[In the above table, H= Chattogram Medical College Hospital (CMC), SE= Standard error, SEM= Standard error mean, BDS= Bangladesh Standards and Testing Institution. Means with different superscript(s) in the same column differ significantly]

Table 4. Nutritional quality of reconstituted milk supplied in different hospitals of CMA

Hospital IDs		Parameter (Mean±SE)					
		Fat%	**Protein%**	**SNF%**	**TS%**	**Titrable Acidity (TA)%**	**Specific Gravity**
B	B_1	1.01±0.044	1.15^{b}±0.020	10.57±0.581	11.7±0.115	0.03^{b} ±0.006	1.039±0.0012
	B_2	5.5^{a}±0.115	3.20±0.115	12.3^{a}±0.115	17.73^{a}±0.203	0.12±0.001	1.042±0.0012
C	C	1.7±0.115	4.25±0.144	9.42±0.012	11.2±0.115	0.24^{a}±0.001	1.034±0.0012
E	E_1	3.5±0.115	2.75±0.144	7.84±0.159	11.3±0.113	0.13±0.012	1.026±0.0006
	E_2	2.7±0.115	5.27±0.145	11.65±0.115	14.3±1.776	0.13±0.002	1.041±0.0006
F	F_1	1.3±0.115	1.35±0.028	9.53±0.124	10.44±0.215	0.03^{b}±0.011	1.034±0.0012
	F_2	0.4±0.088	6.53^{a}±1.155	8.30±0.104	8.18^{b}±0.176	0.06±0.012	1.031±0.0012
	F_3	4.0±0.289	4.90±0.231	8.88±0.188	12.9±0.231	0.04±0.007	1.029±0.0006
	F_4	0.6±0.058	5.10±0.058	8.21±0.159	8.74±0.131	0.04±0.006	1.029±0.0008
	F_5	0.3^{b}±0.058	6.43±1.155	9.43±0.092	9.48±0.148	0.19±0.011	1.028±0.0008
	F_6	3.2±0.058	3.40±0.115	7.67±0.148	10.84±0.159	0.15±0.011	1.029±0.0013
G	G	3.5±0.115	3.40±0.173	11.8±0.173	8.3±0.115	0.18±0.010	1.043^{a}±0.0012
I	I_2	2.4±0.058	3.40±0.058	7.2^{b}±0.115	9.46±0.255	0.12±0.002	1.025^{b}±0.0006
	I_3	3.0±0.058	3.50±0.058	8.93±0.073	11.8±0.192	0.11±0.001	1.031±0.0006
	I_4	3.5±0.058	2.50±0.058	8.11±0.087	11.7±0.058	0.13±0.002	1.027±0.0006
	I_5	2.5±0.058	5.60±0.058	9.10±0.097	11.7±0.058	0.13±0.001	1.032±0.0006
	Average	2.4±0.208	3.92±0.245	9.30±0.219	11.3±0.354	0.11±0.009	1.03±0.0008
	SEM	0.208	0.245	0.219	0.354	0.009	0.0008
	p- value	0.001	0.001	0.001	0.001	0.001	0.001
	Level of Significance	**	**	**	**	**	**

[In the above table, B= Chattogram Maa – O- Shishu Hospital (B_1= F75 Formula (NIDO Fortigrow) B_2= F100 Formula (NIDO Fortigrow), C= CSCR Hospital (Marks Full Cream Milk Powder), E= Chattogram Eye Infirmary Hospital (E_1= Diploma Full Cream Milk Powder without sugar, E_2= Diploma Full Cream Milk Powder without sugar), F= Chattogram Metropolitan Hospital Ltd. (F_1= Nutrifol, F_2= Pentasure DM, F_3= Revit R, F_4=Pentasure, F_5= Pentasure hepatic, F_6= Pentasure Fiber), G= Medical Centre Hospital (Marks Milk Based Diabetic Diet), I= Chattogram Railway Hospital (I_2= Dano Full Cream Milk Powder without sugar, I_3= Dano Full Cream Milk Powder with sugar, I_4= Diploma Full Cream Milk Powder without sugar, I_5= Diploma Full Cream Milk Powder with sugar).). a= Highest value, b= Lowest value, SE= Standard error, SEM= Standard error mean, **= statistically significant ($p<0.01$), Means with different superscript(s) in the same column differ significantly]

The average fat, protein, SNF, TS, TA percentage and specific gravity of pasteurized milk were 1.7±0.115, 4.25±0.144, 7.09±0.054, 8.46±0.258, 0.14±0.001 and 1.025±0.0012 respectively (Table 3). In this case, pasteurized milk was supplied by only one hospital and no p-value was found for pasteurized milk sample.

Among the reconstituted milk samples, the highest fat percentage was 5.5±0.115 in case of hospital B and the lowest was 0.3±0.058 in case of hospital F as shown in table 4. Table 4 illustrates that the highest protein percentage was 6.53±1.155 in hospital F and the lowest was 1.15±0.020 in case of hospital B. In case of SNF, the highest percentage was 12.3±0.115 in hospital B and the lowest was 7.2±0.115 in hospital I. The highest TS percentage was 17.73±0.203 in hospital B and the lowest was 8.18±0.176 in hospital F. The lowest TA percentage was 0.03±0.006 and 0.03±0.011 in case of hospital B and the highest was 0.24±0.001 in case of hospital C shown in table 4. The highest specific gravity was 1.043±0.0012 in hospital G and the lowest was 1.025±0.0006 in case of hospital I. The average value of fat%, protein%, SNF%, TS%, titrable acidity% and specific gravity of reconstituted milk samples was 2.4±0.208, 3.92±0.245, 9.30±0.219, 11.3±0.354, 0.11±0.009 and 1.03±0.0008 respectively. From the results, it was found that all the physicochemical parameters of reconstituted milk samples were differed significantly ($p<0.01$) between hospitals.

4.2 Adulterants in milk

Table 5 shows that presence of common adulterants (water, starch, cane sugar and powder milk) in milk samples were tested in this study. Here, water adulteration test was performed for the raw and pasteurized milk samples. Table 5 shows that all samples (100%) of raw and pasteurized milk irrespective of hospitals were adulterated with water, cane sugar and powder milk besides, starch was detected in 100% raw milk sample. All the samples of pasteurized and reconstituted milk were free from starch but cane sugar was detected in 100% milk samples. Most of the samples were reconstituted milk in this study. Adulteration tests for powder milk and water were not performed in reconstituted milk since reconstituted milk was prepared mixing the powder milk with water.

Table 5. Status of adulterants in all collected milk samples from CMA hospitals

Types of Milk	Types of adulterants					
	Hospital IDs		Water	Starch	Cane Sugar	Powder Milk
Raw	A		+ve	+ ve	+ ve	+ ve
	D		+ ve	+ ve	+ ve	+ ve
	I_1		+ ve	+ ve	+ ve	+ ve
	Total		3	3	3	3
	Percentage (+ve)		100	100	100	100
Pasteurized	H		+ ve	- ve	+ ve	+ ve
	Total		1	1	1	1
	Percentage (+ve)		100	0	100	100
Reconstituted	B	B_1	ND	- ve	+ ve	ND
		B_2	ND	- ve	+ ve	ND
	C	C	ND	- ve	+ ve	ND
	E	E_1	ND	- ve	+ ve	ND
		E_2	ND	- ve	+ ve	ND
	F	F_1	ND	- ve	+ ve	ND
		F_2	ND	- ve	+ ve	ND
		F_3	ND	- ve	+ ve	ND
		F_4	ND	- ve	+ ve	ND
		F_5	ND	- ve	+ ve	ND
		F_6	ND	- ve	+ ve	ND
	G	G	ND	- ve	+ ve	ND
	I	I_2	ND	- ve	+ ve	ND
		I_3	ND	- ve	+ ve	ND
		I_4	ND	- ve	+ ve	ND
		I_5	ND	- ve	+ ve	ND
	Total		16	16	16	16
	Percentage (+ve)		0	0	100	0

[In the above table, A= Chattogram General Hospital, D= Chattogram Diabetic General Hospital, I_1= Chattogram Railway Hospital, H= Chattogram Medical College Hospital, B= Chattogram Maa – O- Shishu Hospital (B_1= F75 Formula (NIDO Fortigrow) B_2= F100 Formula (NIDO Fortigrow), C= CSCR Hospital (Marks Full Cream Milk Powder), E= Chattogram Eye Infirmary Hospital (E_1= Diploma Full Cream Milk Powder without sugar, E_2= Diploma Full Cream Milk Powder without sugar), F= Chattogram Metropolitan Hospital Ltd. (F_1= Nutrifol, F_2= Pentasure DM, F_3= Revit R, F_4=Pentasure, F_5= Pentasure hepatic, F_6= Pentasure Fiber), G= Medical Centre Hospital (Marks Milk Based Diabetic Diet), I= Chattogram Railway Hospital (I_2= Dano Full Cream Milk Powder without sugar, I_3= Dano Full Cream Milk Powder with sugar, I_4= Diploma Full Cream Milk Powder without sugar, I_5= Diploma Full Cream Milk Powder with sugar). +ve= Positive, -ve= Negative, ND= Not done]

Table 6. Prevalence of adulterants in milk samples collected from different hospitals of CMA

Parameters	No. positive	Prevalence (%) (95 % CI)
Water Test	4	20 (0.0573 - 0.437)
Starch Test	3	15 (0.0321 - 0.378)
Cane Sugar Test	20	100 (0.832 – 1.00)
Powder Milk Test	4	20 (0.0573 - 0.437)

Table 6 illustrates that prevalence of water adulteration for the raw and pasteurized milk samples was 20% [95% CI (0.0573 - 0.437)]. Presence of starch in raw milk samples was 15% [95% CI (0.0321 - 0.378)]. Cane sugar was found positive in 100% samples [95% CI (0.832 – 1.00)]. In this study 80% of the total samples were reconstituted milk and 20% of the total samples were raw & pasteurized milk. Since the reconstituted milk is prepared mixing the powder milk with water that's why only raw and pasteurized milk samples were analyzed for powder milk adulteration. Result shows that all of 20% [95% CI (0.0573 - 0.437)] samples of raw and pasteurized milk were positive for powder milk adulteration.

4.3 Preservatives in milk

Table 7 shows that there is no evidence of presence of formalin irrespective of type of milk and hospitals. Among the raw milk samples, hydrogen peroxide was found positive in 100% samples whereas carbonate or bi- carbonate was found to be negative for all the raw samples. Hydrogen peroxide and carbonate or bi- carbonate were found to be negative in both pasteurized and reconstituted milk samples. The reconstituted milk samples were free from any of the tested four preservatives.

Table 7. Status of added preservatives in all collected milk samples from CMA hospitals

Types of milk	Types of preservatives				
	Hospital IDs		Formalin	Hydrogen peroxide	Carbonate or bi-carbonate
Raw	A		- ve	+ ve	- ve
	D		- ve	+ ve	- ve
	I_1		- ve	+ ve	- ve
	Total		3	3	3
	Percentage (+ve)		0	100	0
Pasteurized	H		- ve	- ve	- ve
	Total		1	1	1
	Percentage (+ve)		0	0	0
Reconstituted	B	B_1	- ve	- ve	- ve
		B_2	- ve	- ve	- ve
	C	C	- ve	- ve	- ve
	E	E_1	- ve	- ve	- ve
		E_2	- ve	- ve	- ve
	F	F_1	- ve	- ve	- ve
		F_2	- ve	- ve	- ve
		F_3	- ve	- ve	- ve
		F_4	- ve	- ve	- ve
		F_5	- ve	- ve	- ve
		F_6	- ve	- ve	- ve
	G	G	- ve	- ve	- ve
	I	I_2	- ve	- ve	- ve
		I_3	- ve	- ve	- ve
		I_4	- ve	- ve	- ve
		I_5	- ve	- ve	- ve
	Total		16	16	16
	Percentage (+ve)		0	0	0

[In the above table, A= Chattogram General Hospital, D= Chattogram Diabetic General Hospital, I_1= Chattogram Railway Hospital, H= Chattogram Medical College Hospital, B= Chattogram Maa – O- Shishu Hospital (B_1= F75 Formula (NIDO Fortigrow) B_2= F100 Formula (NIDO Fortigrow), C= CSCR Hospital (Marks Full Cream Milk Powder), E= Chattogram Eye Infirmary Hospital (E_1= Diploma Full Cream Milk Powder without sugar, E_2= Diploma Full Cream Milk Powder without sugar), F= Chattogram Metropolitan Hospital Ltd. (F_1= Nutrifol, F_2= Pentasure DM, F_3= Revit R, F_4=Pentasure, F_5= Pentasure hepatic, F_6= Pentasure Fiber), G= Medical Centre Hospital (Marks Milk Based Diabetic Diet), I= Chattogram Railway Hospital (I_2= Dano Full Cream Milk Powder without sugar, I_3= Dano Full Cream Milk Powder with sugar, I_4= Diploma Full Cream Milk Powder without sugar, I_5= Diploma Full Cream Milk Powder with sugar). +ve= Positive, -ve= Negative]

Table 8. Prevalence of preservatives in milk samples collected from different hospitals of CMA

Parameters	No. positive	Prevalence (%) (95 % CI)
Formalin Test	0	0 (0.0013 - 0.2487)
Hydrogen Peroxide Test	3	15 (0.0321 - 0.378)
Carbonate or Bi-Carbonate Test	0	0 (0.0013 - 0.2487)

Table 8 illustrates that the prevalence of formalin for all the collected milk samples was 0% [95% CI (0.0013 - 0.2487)]. Presence of hydrogen peroxide in raw milk samples was 15% [95% (0.0321 - 0.378)]. Carbonate or bi- carbonate was found to be negative [0% [95% CI (0.0013 - 0.2487)]] for all the collected milk samples.

4.4 Microbiological Evaluation

4.4.1 Total Viable Bacterial Count (TVC)

Table 9 reveals that, the highest TVC was 1.50×10^6 CFU/ml in hospital A in case of raw milk sample and the lowest was 1.25×10^4 CFU/ml found in case of hospital F in the reconstituted milk samples.

4.4.2 Total Coliform Count (TCC)

Among all the collected milk samples, the highest coliform bacterial count was 1.30×10^6 CFU/ml in the raw milk sample of hospital A and the lowest count was 1.2×10^2 CFU/ml that was found in hospital C in case of reconstituted milk sample. In this study, total coliform count was negative in all pasteurized milk samples (Table 9).

4.4.3 *Escherichia coli*

None of the collected milk samples have produced characteristic colony with greenish metallic sheen on EMB agar. Table 10 shows that all the collected milk samples were found negative for *E. coli*.

Table 9. Microbial analysis of milk collected from hospitals of CMA

Hospital IDs		Total Viable Bacterial Count (CFU/ml)	Total Coliform Count (CFU/ml)	*Escherichia coli*	*Staphylococcus aureus*
A	A	$1.50\times10^{6}{}_{a}$	$1.30\times10^{6}{}_{a}$	Nil	Positive
B	B_1	3.05×10^{5}	3.00×10^{2}	Nil	Positive
	B_2	6.05×10^{5}	6.00×10^{3}	Nil	Positive
C	C	9.40×10^{4}	$1.20\times10^{2}{}_{b}$	Nil	Positive
D	D	1.70×10^{5}	1.10×10^{5}	Nil	Positive
E	E_1	1.25×10^{4}	1.25×10^{4}	Nil	Positive
	E_2	4.30×10^{5}	1.50×10^{3}	Nil	Positive
F	F_1	1.25×10^{5}	1.25×10^{3}	Nil	Positive
	F_2	1.65×10^{5}	1.30×10^{2}	Nil	Positive
	F_3	1.75×10^{4}	1.45×10^{4}	Nil	Positive
	F_4	3.50×10^{4}	2.00×10^{2}	Nil	Positive
	F_5	5.50×10^{5}	1.50×10^{3}	Nil	Positive
	F_6	$1.25\times10^{4}{}_{b}$	1.60×10^{2}	Nil	Positive
G	G	7.50×10^{5}	1.25×10^{3}	Nil	Positive
H	H	1.31×10^{5}	Nil	Nil	Positive
I	I_1	6.20×10^{5}	1.90×10^{4}	Nil	Positive
	I_2	1.50×10^{5}	1.30×10^{5}	Nil	Positive
	I_3	1.25×10^{5}	1.70×10^{3}	Nil	Positive
	I_4	1.75×10^{4}	1.65×10^{4}	Nil	Positive
	I_5	4.10×10^{5}	5.2×10^{3}	Nil	Positive

[In the above table, A= Chattogram General Hospital, D= Chattogram Diabetic General Hospital, I_1= Chattogram Railway Hospital, H= Chattogram Medical College Hospital, B= Chattogram Maa – O-Shishu Hospital (B_1= F75 Formula (NIDO Fortigrow) B_2= F100 Formula (NIDO Fortigrow), C= CSCR Hospital (Marks Full Cream Milk Powder), E= Chattogram Eye Infirmary Hospital (E_1= Diploma Full Cream Milk Powder without sugar, E_2= Diploma Full Cream Milk Powder without sugar), F= Chattogram Metropolitan Hospital Ltd. (F_1= Nutrifol, F_2= Pentasure DM, F_3= Revit R, F_4=Pentasure, F_5= Pentasure hepatic, F_6= Pentasure Fiber), G= Medical Centre Hospital (Marks Milk Based Diabetic Diet), I= Chattogram Railway Hospital (I_2= Dano Full Cream Milk Powder without sugar, I_3= Dano Full Cream Milk Powder with sugar, I_4= Diploma Full Cream Milk Powder without sugar, I_5= Diploma Full Cream Milk Powder with sugar). a= Highest value, b= Lowest value. CFU= Colony Forming Unit]

4.4.4 Prevalence of *Staphylococcus aureus*

All the collected milk samples were found positive [100% [95% CI (0.832 – 1.00)]] for *Staphylococcus aureus*. *S. aureus* was confirmed by observing the cultural and morphological characteristics of the isolates grown on specific culture media (Table 10). Among 20 positive isolates of *S. aureus*, ten (10) samples [50% [95% CI (0.2719 - 0.7280)]] were found positive for coagulase test.

Table 10. Prevalence of coagulase positive *S. aureus*

Total no. of samples	**No. of positive isolates with prevalence%**	**No. of coagulase positive *S. aureus***	**Prevalence of coagulase positive isolates (%) (95 % CI)**
20	20, 100% [95% CI (0.832 – 1.00)]]	10	50 (0.2719 - 0.7280)

CHAPTER – V

Discussion

Chapter V: Discussion

The study was intended for the evaluation of nutritional status and analysis of microbial hazards in the milk consumed by the patients in different hospitals of CMA.

5.1 Physicochemical Properties of milk

5.1.1 Fat

The present study reveals that average fat percentage in raw milk samples was ranged from 0.5 to 2.2 which are greatly disagreed with the findings of Debnath *et al*., (2009) who demonstrated that the butter fat of milk from different sources from Chattogram Metropolitan Area were ranged from 3.52 to 4.01%. In this study, average fat percentage was lowered might be due to the addition of water in raw milk in different hospitals of CMA. Graf (1976) suggested that The U.S. Public Health Service (USPHS) Milk Ordinance and Code also recommended a minimum of 3.25% butterfat in farm milk. However, in this study, average fat percentage of collected raw milk samples was 1.1. This result is disagreed with USPHS Standards. According to BDS (2002), the minimum fat content of pasteurized milk should be 3.5%. In present study, the fat content of pasteurized milk samples were recorded 1.7%, which was greatly differed from the Bangladesh Standards. From the results, it was also seen that among the reconstituted milk samples, the average fat percentage was 2.4%. In this study, average fat percentage was decreased might be due to the addition of water during reconstitution for patients in different hospitals of CMA (Harding, 1995; Nickerson, 1995).

5.1.2 Protein

The protein content of all the raw milk samples was ranged from 3.5 to 4.43 which were slightly higher than the values of Ramasamy et al., 1999 found that the protein content of cow's milk varied from 3.22% to 3.92 %.In this study, average protein content of raw milk was 4.01 which was dissimilar with the study of Lingathurai *et al*., (2009), found that the standard protein content of raw milk showed 3.77%. According to BDS (2002), protein content of pasteurized milk is not lower than 3.3%. However, in this study, it was found that average protein content of pasteurized milk was 4.25% which was disagreed with the study of Hossain et al., (2011) observed highest protein content (3.35%) followed by 3.49% and 3.51% protein in three pasteurized milk samples available in Bangladesh.

The protein content of pasteurized milk is mostly affected by heating and subsequent storage condition (Fox et al., 1998).

From this study, it was also resembled that protein percentage of reconstituted milk samples was ranged from 1.15 to 6.53. The difference between higher and lower limit of protein content in milk was remarkable. The average protein percentage of reconstituted milk samples was 3.92. It might be due to not following the recommended amount of powder milk to be mixed with water.

5.1.3 SNF (Solids-not-fat)

From the results it was found that the average SNF% of the raw milk samples was found 7.41 which was somewhat lower than the findings of Debnath *et al*., (2009), who reported that SNF% of farm produced milk, vendor supplied farm milk, vendor supplied rural milk and brand market milk were 8.33%, 7.98%, 7.85%, 8.2%, respectively. Addition of water might be the cause of lower SNF percentage. The pasteurized milk contained 7.09% which was slightly lower than the recommended standard of BDS (8% SNF). On the other hand, most of the milk samples of this study were reconstituted milk, SNF% ranged from 7.2 to 12.3 were estimated in the collected samples. The differences of the SNF percentage in reconstituted milk might be due to not following the proper guidelines of adding water during reconstitution of powder milk by the hospitals authority. The average SNF% of reconstituted milk samples was 9.30.

5.1.4 Total Solids (TS)

In present study, it is noticed that average percentage of total solids of raw milk samples was 8.44 which was a bit lower than the standard value of FAO. TS content can be found lower if water is added to the milk. The average percentage of total solids of pasteurized milk samples was 8.46 which were also lower than the standard of BDS (11.50%). In this study, the major source of sample was reconstituted milk, where the average TS% was 11.3 which was very remarkable and differed significantly ($p<0.01$).

5.1.5 Titrable Acidity (TA)

Gould and Jensen (1944) recommended that titrable acidity used as an indicator of quality for milk. It is expressed as percent of lactic acid. Fresh milk does not contain any appreciable amount of lactic acid and therefore an increase in acidity is a rough measure of its age and bacterial activity (O'Mahony, 1988; Lampart, 1947). Popescu and Angel (2009) stated that high quality milk has to have less than 0.14% acidity. Results revealed that the average acidity percentage of raw milk samples was 0.18. This result is disagreed with the study of Popescu and Angel (2009).

The average acidity% of pasteurized milk samples was 0.14. The acidity% was within the limit as recommended by BDS, 2002 and it indicated that the cool chain was maintained properly for storing the milk. In another study done by Elmagli *et al*. (2006) was found a greater range of acidity (0.14 to 0.16%) in pasteurized milk which was similar to the findings of present study.

In this study, the average % of lactic acid of reconstituted milk samples was 0.11. Acidity rapidly develops in reconstituted and recombined milk because the lactic acid producing bacteria grows well in these two medium due to easy access to lactose. In the present study, acidity% of reconstituted milk was higher due to growth of lactic acid producing bacteria (Uddin et al., 2013).

5.1.6 Specific Gravity

The average specific gravity of the raw milk samples was ranged from 1.026 which was supported by Eckles *et al*., (1951), Islam *et al*., (1993) and Debnath *et al*., (2009). Adulteration of milk by adding water might decrease the specific gravity of raw milk in this study. The average specific gravity of pasteurized milk was 1.025 which was lower than as recommended (1.028 to 1.032) by BDS (2002). So, it can be said that the pasteurized milk was not standardized properly by the manufacturer. The specific gravity was 1.03 among the reconstituted milk samples which was higher than the study of Lateef *et al*., (2009) who have shown that the value was 1.02 for milk marketed at the canteens of various hospitals located in the city of Faisalabad, Pakistan. The differences of specific gravity in reconstituted milk might be due to not following the proper guidelines of adding water during reconstitution of powder milk by the hospitals authority.

5.2 Adulterants in milk

Water adulteration was found in 100% milk samples in case of raw and pasteurized. In this study, starch was detected as an adulterant in all the raw milk samples which was disagreed with the findings of Islam *et al*., (2013) who found that no milk was adulterated with starch. Here, all the raw and pasteurized milk samples showed positive result for the presence of cane sugar. Islam *et al*., (2018) found that the most common adulterant in our dairy industry is cane sugar. SNF content of milk is increased by using this adulterant. Moreover, only 0.2% addition of cane sugar can increase lactometer reading by one degree at 60 °F. Monem (2012) observed that cane sugar is added into milk to increase the solids-not-fat (SNF) content of milk after addition of water. According to Mamun *et al*., (2016) the addition of cane sugar in milk helps to improve the taste of diluted raw milk.

In the present study, it is found that all the raw and pasteurized milk samples were adulterated with powder milk that might contribute to increase the SNF and TS content of milk. Standardization is the common steps for market milk processing and it is done either by adding skim milk or powder milk. Presence of milk powder in pasteurized milk might be due to standardization process (Debnath *et al*., 2009).

5.4 Preservatives in milk

The present study revealed that all the collected milk samples of different hospitals were free from formalin and carbonate and bicarbonate, but in the study of Kamel (2000), reveals that 30% of market milk samples were positive for formalin which is greatly disagreed with the present findings. Moreover, Debnath *et al*., (2009) and Das *et al*., (2009) found formalin as the only added preservative in raw milk of CMA, these findings also stated against the finding of present study. Hydrogen peroxide was found positive in 100% raw milk sample which is not supported by the results of Abdel-Hameid (2002) and Wahba and Korashy (2006). The probable cause of this dissimilar result might be due to the collected milk samples was used as the nutritious supplement for the patient in selected hospitals of CMA where public health issues were closely monitored. So hospitals authority was ensured the reliable suppliers of raw milk.

5.5 Microbiological analysis

5.5.1 Total Viable Bacterial Count (TVC)

In a sample, the total viable bacterial count is the number of bacteria which can grow and form countable colonies on nutrient agar medium (i.e.; Plate Count Agar) after being incubated at 37°C for overnight.

In this study, all the raw milk samples had a bacterial load ranged from 6.20×10^5 to 1.5×10^6 CFU/ml, which was lower than the range (4×10^6 to 2.7×10^7) CFU/ml found by Lee *et al*., (1996). Arenas *et al*., (2004) reported that the total number of micro-organisms in pooled raw milk was 5.5×10^6 CFU/ml. This result is also higher than the present findings. Poor hygienic condition is one of the major causes of high bacterial load in raw milk.

Iknomov *et al*., (1956) reported that the total bacterial count was ranged from 1.25×10^5 to 9.0×10^6 CFU/ml of milk depending on milking techniques and cleanliness. The TVC of the pasteurized milk sample was 1.31×10^5 CFU/ml which was significantly higher than the recommended BDS and USPHS standards where the highest acceptable count is 20,000 CFU/ml **(**BDS, 2002; Jay, 2003). The reasons for high bacterial count in the pasteurized milk might be due to high bacterial load in raw milk, defective pasteurization, presence of thermoduric bacteria, post-pasteurization contamination, improper storage and marketing temperature etc.

Furthermore, in the present study, the TVC (total viable bacterial count) of the reconstituted milk samples was ranged from 7.50×10^5 to 1.25×10^4 CFU/ml, which may results due to the addition of poor quality water to dissolve the milk, lactic acid producing bacteria multiplicates rapidly due to easy availability of lactose (Uddin *et al*., 2013).

5.5.2 Total Coliform Count (TCC)

In the present study, it is noticed that the coliform count was ranged from 1.30×10^6 to 1.10×10^3 CFU/ml in all the raw milk samples which was higher than that obtained by Srari *et al*., (2006) who found that the TCC (total coliform count) of raw milk was less than 30 to 2.08×10^7 CFU/ml. The higher coliform count indicates poor sanitary practices of dairy farm and processing unit.

It may results from irregular bathing of animal, feeding system of animal in low land, muddy cow yard, unsanitary milking utensils, contamination of body surface by feces, poor personnel hygiene etc. (Khaton *et al*., 2014). Godefay and Molla (2000) and Uddin *et al*., (2011) found coliform counts above 1×10^4 CFU/ml which was somewhat similar to the present findings.

According to the standard of BDS, the pasteurized milk should contain less than 10 CFU/ml Coliform. According to USPHS, samples having more than 10 CFU/ml will not be graded as 'Grade A' pasteurized milk (Jay, 2003). In this study the TCC was found nil in the pasteurized milk samples which indicated that the temperature-time combination for pasteurization was perfect. Coliform bacteria are supposed to be absent in pasteurized milk as they can't survive at pasteurization temperature.

Among all the reconstituted milk samples, TCC ranged from 1.30×10^5 to 1.05×10^2 was considerably higher that might be due to the poor water quality that was used to liquefy the powder milk. Arum *et al*., (1970) said that the incidence of coliforms in any processed food is related to the unhygienic manufacturing techniques including poor plant sanitation and post processing contamination which was supported by the present findings. According to Bille *et al*., (2009), the absence of lower numbers of coliforms in milk provides an index of hygienic standard used in the production of milk, as clean udder and teats can contribute to the absence of coliforms from a variety of sources such as manure, soil, food, personnel and even water.

5.5.3 *Escherichia coli*

The presence of coliform bacteria, such as *E. coli*, in milk is a common indicator because their presence in food indicates some form of fecal contamination. From this study, it was found that all the collected milk samples were found to be negative for *E. coli*. This result is greatly agreed with BDS standards (BDS, 2000).

5.5.3 *Staphylococcus aureus*

In this study, the results indicated that all the milk samples were found to be positive for *S. aureus* that means prevalence rate is 100% [95% CI (0.832 – 1.00)]. To evaluate the degree of contamination of milk with *S. aureus* obtained from communal and commercial farms, various studies have been conducted.

In most cases, milk of the animals suffering from subclinical mastitis contains *S. aureus*. Zafolon *et al*., (2008) studied at Nova Odesa, São Paulo; reported that the prevalence of *S. aureus* was 54.4% which is lower than the results obtained in this study.

The comparatively lower prevalence of *staphylococcal* count was also found by Ekici *et al*., (2004), Bendahou *et al*., (2008) & Lingathurai and Vellathurai, (2010) which were 18.18%, 40% and 61.7% respectively. This higher *staphylococcal* count in milk samples could be the consequence of poor management and improper hygienic practices during farming, milking, transportation, processing and preparation of the samples (Zakary *et al*., 2011).

In the present study, it was shown that 50% of the samples were found coagulase positive for the presence of *S. aureus* which was significantly higher than the result (15%) found by linage *et al*., (2012), where they isolated coagulase positive *S. aureus* from tank and silo of ewe milk.

CHAPTER – VI

Conclusion

Chapter VI: Conclusion

Milk is one of the valuable foods that readily digests and absorbs into the human system. It consists of all essential nutrients for the proper growth and maintenance of the body. That is why milk is an ideal food for all age groups and specially, for patients. The quality of milk may deteriorate due to malpractices, unhygienic production, careless handling and storage. In the present study milk samples (raw, pasteurized and reconstituted) supplied at different hospitals of CMA were tested. The nutritional and physicochemical quality of reconstituted milk in the study was comparatively better compared to raw and pasteurized milk. On the other hand, the microbial quality of all three types of milk was found higher than the recommended level. Adulteration was the common scenario in all three types of milk sample. Moreover, some unauthorized preservatives were detected in raw milk samples, which is an alarming issue for the patients. The hospital authorities should look into the matter seriously in order to ensure the supply of safe milk to the admitted patients.

CHAPTER – VII

Recommendations and Future Perspectives

Chapter VII: Recommendations and Future Perspectives

The main objective of this research was to investigate the nutritional status and microbial quality of milk samples collected from different govt. and non- govt. hospitals of Chattogram Metropolitan Area. According to this study, a number of recommendations for future research are given below-

- Study with a large number of samples and more detailed examination of the milk is highly recommended in future.
- Further study needed regarding the chemical (adulteration) and microbiological quality for all types of milk supplied at the hospitals.
- Hospital authority should come forward to ensure the supply of safe milk to the patients, which can be done with the collaboration of the Department of Dairy and Poultry Science, CVASU.
- Proper heat treatment and use of safe drinking water must be ensured prior to supply raw and reconstituted milk respectively.
- Till now there is no research published on the content of nutrition status & microbial quality of milk supplied at different hospitals available in Chattogram Metropolitan Area. Future research work on this will be a new dimension to control food borne diseases and to lessen the public health hazards in Bangladesh.

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Appendices

Thesis for MS in Applied Human Nutrition and Dietetics

Appendices

Appendix A

Photo Gallery of Hospitals

Figure 27: Chattogram General Hospital

Figure 28: Chattogram Maa -O- Shishu Hospital

Figure 29: CSCR Hospital

Figure 30: Chattogram Diabetic General Hospital

Figure 31: Chattogram Eye Infirmary Hospital

Figure 32: Chattogram Metropolitan Hospital Ltd.

Figure 33: Medical Centre Hospital

Figure 34: Chattogram Medical College Hospital

Figure 35: Chattogram Railway Hospital

Appendix B

Photo Gallery of Samples

Figure 36: Raw milk (I_1)

Figure 37: Powder milk used for reconstitution (B_1 and B_2)

Figure 38: Powder milk used for reconstitution

(C)

Figure 39: Powder milk used for reconstitution

(E_1, E_2, I_4, I_5)

Figure 40: Powder milk used for reconstitution (F_1)

Figure 41: Powder milk used for reconstitution (F_2)

Figure 42: Powder milk used for reconstitution (F_3)

Figure 43: Powder milk used for reconstitution (F_4)

Figure 44: Powder milk used for reconstitution (F_5)

Figure 45: Powder milk used for reconstitution (F_6)

Figure 46: Powder milk used for reconstitution (G)

Figure 47: Pasteurized milk (H)

Figure 48: Powder milk used for reconstitution (I_2, I_3)

Brief bio-data of the student

Afra Binte Iftekhar, completed B.Sc. (Hons.) in Food Science and Technology from the Faculty of Food Science and Technology of Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh with CGPA 3.67 out of 4.00. Now, she is a candidate for the degree of MS in Applied Human Nutrition and Dietetics under Department of Applied Food Science and Nutrition, Chattogram Veterinary and Animal Sciences University (CVASU). She has immense interest to work in public health perspective such as to evaluate the chemical and microbial quality of milk supplied at different hospitals of Chattogram Metropolitan Area.