IDENTIFICATION OF BACTERIA AND THEIR LOADS ISOLATED FROM DIFFERENT BRANDS OF ICE CREAM

A THESIS
BY

MD. KAMRUL HASAN
Registration No. 1105114
Semester : July-December , 2012

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

FACULTY OF VETERINARY AND ANIMAL SCIENCE

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY

UNIVERSITY , DINAJPUR , BANGLADESH

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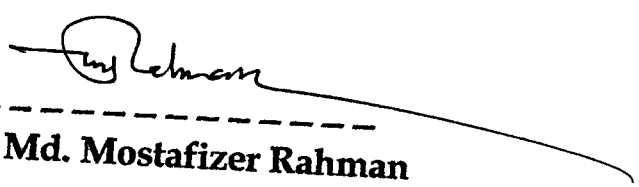

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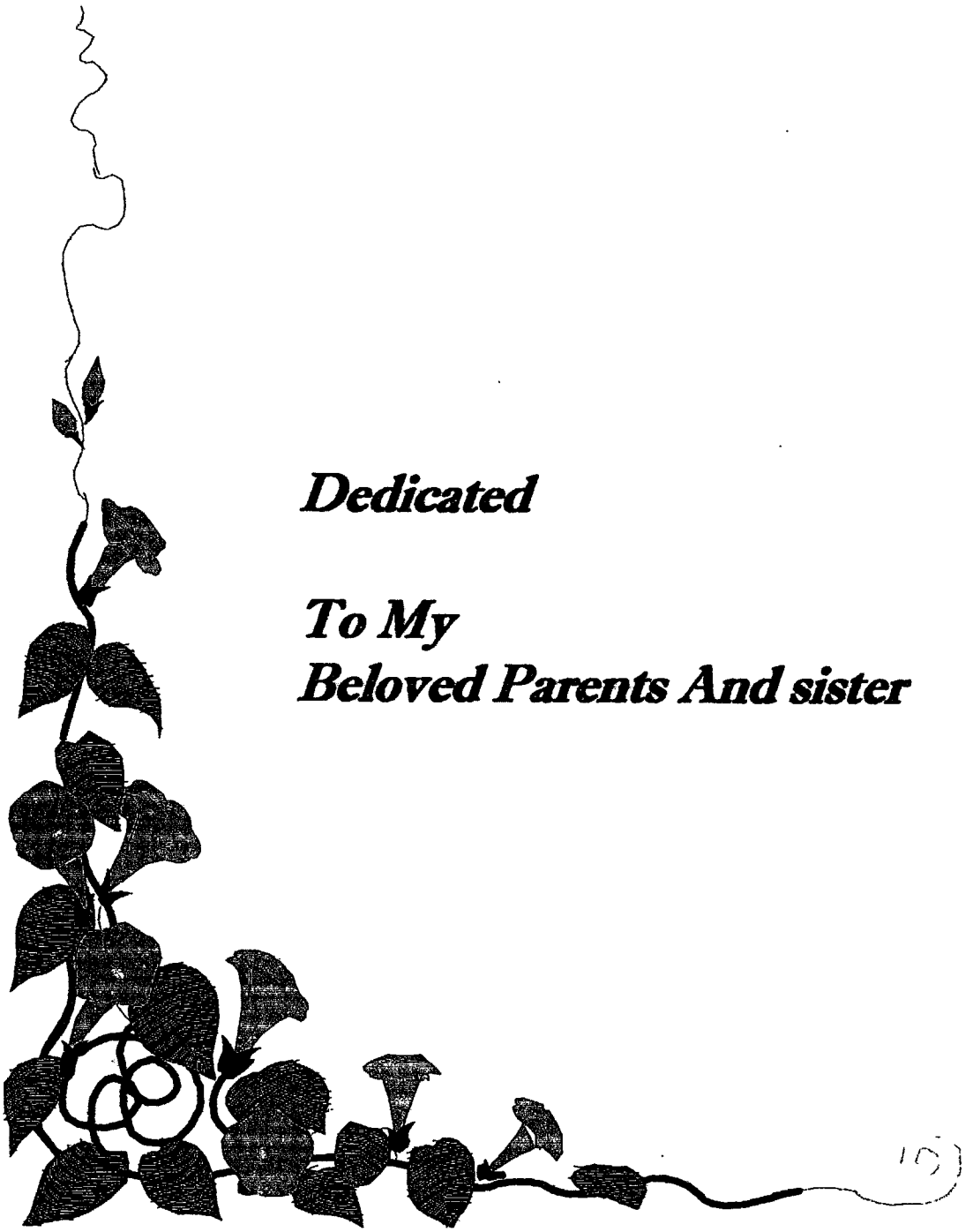

Dedicated

To My
Beloved Parents And sister

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"In the Name of Allah, the most Beneficent, the Most Merciful. All the praises and thanks be to Allah, the Lord of the Alamin (mankind, jinns and all that exists)." - Surnh AI- Fatihah (1-2).

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ABSTRACT

A study was conducted to identify the organisms in ice cream manufactured by three different plants namely Igloo, Polar and Kwality sold in retail stores in Dinajpur town, Bangladesh. The samples were analyzed to determine the hygienic status of the ice creams and also for the total viable count, presence of Gram positive and Gram negative pathogenic bacteria.Three cups ice cream samples each of three popular varieties including Igloo, Polar and Kwality were collected from different retail stores in Dinajpur city. The bacteriological analyses were done at the Microbiology Laboratory of Hajee Mohammad Danesh Science and Technology University, Dinajpur. During July to December -2012.

Total viable count (TVC) was performed according to the American Public Health Association, using plate count agar medium for TVC and Eosine methylene blue (EMB) agar media for total *E. coli* count and Staphylococcus agar no. 110 for total staphylococcus count.

The average TVC for Kwality ice cream was lower (8.53×10^3 CFU/ml) in comparison to Polar ice cream (1.39×10^4 CFU/ml). It was apparent from the present study that the bacterial load of the ice cream samples of the three manufacturers were within the acceptable limit of public health safety, since the count was well below the acceptable limit.

It was found that the highest extent of microbial contamination and proliferation of viable bacteria occurred in Polar ice cream. The average *E. coli* counts obtained from the study was in Igloo 9.26×10^3 CFU/ml (log 4.0), in Polar 1.14×10^3 CFU/ml (log 3.0) and in Kwality 7.95×10^2 CFU/ml (log 2.9) . The presence of numbers of *E. coli* in Igloo ice cream was little bit high indicated the poor hygienic practices during manufacture, post process contamination and unsatisfactory transportation. Statistically the *E. coli* were more closely related to total viable counts than the staphylococcal counts. The average staphylococcal counts in the samples of Igloo, Polar and Kwality were 2.60×10^3CFU/ml (log 3.4), 0.0 CFU/ml (log 0.0), and 0.0 CFU/ml (log 0.0), respectively.

The results demonstrated that kwality ice creams are of the superior quality product in respect of sanitary condition.

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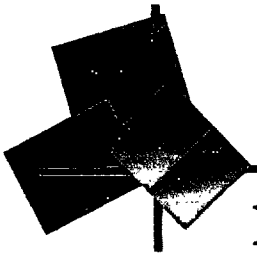
LIST OF ABBREVIATIONS AND SYMBOLS

LIST OF ABBREVIATIONS AND SYMBOLS		
-	=	Negative
% / < >	= = = =	Percentage Per Less than Greater than
+	=	Positive
µg	=	Microgram
µl	=	Microliter
^0C	=	Degree celsius
CFU	=	Colony forming units
Dx	=	Dextrose
E. Coli	=	*Escherichia coli*
e.g.	=	Example
EMB	=	Eosin methylene blue
et al.	=	Associated
etc	=	Etcetera
Fig.	=	Figure
gm	=	Grams
H_2S	=	Hydrogen sulfied
hrs	=	Hours
HSTU	=	Hajee Mohammad Danesh Science and Technology University
Ib	=	Pound
Kg	=	Kilogram
KOH	=	Potassium hydroxide
L	=	Lactose

Log	=	logarithms
Ltd	=	Limited
MC	=	MacConkey
mg	=	Milligram
min	=	Minutes
MIU	=	Motility Indole Urease
ml	=	Milliliter
ML	=	Maltose
mm	=	Millimeter
MN	=	Mannitol
MR	=	Methyl Red
n	=	Number
NA	=	Nutrient agar
NB	=	Nutrient broth
No.	=	Number
PBS	=	Phosphate buffered saline
R	=	Resistant
S	=	Sucrose
Sec	=	Seconds
SL.	=	Serial
Sq	=	Square
TEC	=	Total *E. coli* count
TSC	=	Total *Staphylococcal* count.
TSI	=	Triple sugar iron
TVC	=	Total viable count
VP	=	Voges Proskauer



Chapter 1

INTRODUCTION

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INTRODUCTION

Ice cream is a delicious, wholesome, nutritious frozen dairy food. It is made of milk, sweet cream, skim milk, condensed milk or other concentrated dairy products or a combination of these with added sugar, flavoring and stabi-lizer with or without color and with incorporation of air during the freezing process. Ice cream is undoubtedly one of most popular and favorite food product in Bangladesh among children and adults especially during summer season. Several brands of ice cream in variety of flavors have been marketed here.

Ice cream, a milk based product, is good medium for microbial growth due to high nutrient value, almost neutral pH value and long storage duration. Quality of ice cream depends on both extrinsic factors that include manufacture procedure, and intrinsic factors that include proportion of ingredients used. The biological agent contaminated with in food are traced to ingredient added post pasteurization and environmental factors such as compressed air fault in storage tank, cracks in the plant and sanitary quantity of ice-cream packaging materials. Primary sources of microbial contamination to ice cream include water and raw milk whereas secondary sources include flavouring agents, utensils and handling. At many points during production, transportation and storage, milk can be contaminated with biological agents. Many psychrophiles and psychrotolerant microorganisms like *Listeria monocytogens* (WHO 1998), *Staphylococcus aureus* (Massa *et al.* 1989), *Bacillus* (Torkar & Mozina 2000), *Salmonella* (Vought & Tatini 1998), *Shigella, Streptococus, Pseudomonas, Campylobacter, Brucella* , *E. coli* and other bacteria are generally present in ice cream.

The Centre for Food Safety (CFS) announced the findings of a targeted food surveillance project to assess the microbiological quality of ice-cream and frozen confections today . The overall satisfactory rate remains high at 99.5 per cent, same as that of last year.A total of 1,100 ice-cream and frozen confection samples, including soft ice-cream, sundaes, ice-cream in original wrapper, ice-cream scoops and popsicles were collected from over 400 food factories, mobile vans, supermarkets, restaurants and retail outlets for testing of pathogens (e.g. *Salmonella* and *Staphylococcus aureus*) and hygiene indicators (coliform organisms and total bacterial count), a spokesman for the CFS said.

Hazard Analysis Critical Control Points (HACCP) is a systematic approach used in the food industry for the identification, assessment, and control of biological, chemical, and physical hazards providing an effective way to advance food quality/safety, focusing on preventing hazards and improving processes (Swanson & Anderson 2000). The effectiveness of HACCP depends on the correct application of its principles, combined with other programs (prerequisites), such as Good Manufacturing Practices (GMPs), Good Hygiene Practices (GHPs), and Sanitation Standard Operating Procedures (SSOPs) (Roberto *et al.* 2006). For a successful food quality/safety control, prerequisite programs should be developed.

HACCP implementation generally improves the microbiological quality of food products (Soriano *et al.* 2002). The evaluation of the microbiological testing results can give important information not only on the quality of the foodstuff at a specific stage of its processing, but on the overall efficiency of HACCP design and implementation as well (Kokkinakis & Fragkiadakis 2007).

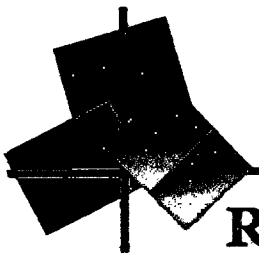
The microbiological quality of ice cream can be low, as it is a good growth-medium for microbes due to its nutrients (lactose, proteins, etc) and to its almost neutral pH

of 6–7 (Kanbakan *et al.* 2004). Time-dependent heating during the ice cream production reduces largely the vegetative forms of the microorganisms. On the other hand, spore bearing microorganisms may well pose risks through consumption of this kind of milk products. Furthermore, the presence of pathogens in ice cream samples is mostly due to tools and equipments, water, workers, environment, packaging materials and contaminations during the transportation and distribution of ice cream.

In developed countries ice cream receives quality control measures to increase its shelf life as well as to prevent potential threat of public health. Bangladesh is still backward in this respect. Due to non enforcement of inspection act and lack of maintenance of standard relation to hygienic quality of ice cream , the consumers of this country are deprived of getting quality ice cream in our country. Some commercial company have been marketed ice cream in the local market. The microbiological status of ice cream for public health significance in Germany is known, but such type of investigation is not known in Bangladesh. It is essential to create awareness among the consumers about the microbiological quality ice cream. Therefore the present work was conducted to determine the total bacterial load in some popular varieties of ice cream samples.

Bearing in mind the above facts the present study was undertaken with the following specific objectives:

- To investigate the microbiological quality of several ice cream samples.
- To performe total viable count (TVC) of organisms ,Total E. coli count (TEC) and total *Staphylococcal* count (TSC) from several ice cream samples.
- Isolation and identification of *E. coli* and *Staphylococcus* from ice cream samples.



Chapter 2

REVIEW OF LITERATUREs

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The purpose of this chapter is to provide a selective review of the research works accomplished in relation to the present study. Literatures on isolation and identification of organisms from ice cream related to this study have been reviewed under the following headings.

Ferraz *et al.* (2012) reported the effect of different overrun levels on the sensory acceptance and survival of probiotic bacteria in ice cream. Vanilla ice creams supplemented with *Lactobacillus acidophilus* were processed with overruns of 45%, 60%, and 90%. Viable probiotic bacterial counts and sensory acceptance were assessed. All the ice creams presented a minimum count of 6 log CFU/g at the end of 60 day of frozen storage. However, higher overrun levels negatively influenced cell viability, being reported a decrease of 2 log CFU/g for the 90% overrun treatment. In addition, it was not reported an influence about acceptability with respect to appearance, aroma, and taste of the ice creams ($P > 0.05$).

Buvens *et al.* (2011) confirmed O145:H28 infection for three hemolytic uremic syndrome patients, one of whom was coinfected with O26:H11. The epidemiological and laboratory investigations revealed ice cream as the most likely source of the outbreak. The ice cream was produced at a local dairy farm using pasteurized milk. VTEC of both serotypes with indistinguishable pulsed-field gel electrophoresis patterns were isolated from patients, ice cream, and environmental samples. Quantitative analysis of the ice cream indicated concentrations of 2.4 and 0.03 CFU/g for VTEC O145 and O26, respectively. Virulence typing revealed that the repertoire of virulence genes carried by the O145:H28 outbreak strain was comparable to that of O157 VTEC and more exhaustive as compared to the O26:H11 outbreak strain and nonrelated clinical strains belonging to these serotypes.

Ozturk *et al.* (2010) showed the effects of the incorporation of some herbal teas at different concentrations into the ice cream mix on the population of *Listeria monocytogenes* were studied using Taguchi method. The ice cream mix samples flavored with herbal teas were prepared using green tea and sage at different concentrations. Afterward, fresh culture of *L. monocytogenes* was inoculated into the samples and the *L. monocytogenes* was counted at different storage periods. Taguchi method was used for experimental design and analysis. In addition, some physicochemical properties of samples were examined. Results suggested that there was some effect, although little, on the population of *L. monocytogenes* when herbal tea was incorporated into the ice cream mix. Additionally, the use of herbal tea caused a decrease in the pH values of the samples and significant changes in the color values.

Schulz *et al.* (2010) examined 809 samples of ice cream from different sources were investigated by using cultural methods for the presence of presumptive *Bacillus cereus*. Isolates from culture-positive samples were examined with a real-time PCR assay targeting a region of the cereulide synthetase gene (ces) that is highly specific for emetic *B. cereus* strains. The samples were collected from ice cream parlors and restaurants that produced their own ice cream and from international commercial ice cream companies in different regions of Bavaria during the summer of 2008. Presumptive *B. cereus* was found in 508 (62.7%) ice cream samples and 24 (4.7%) of the isolates had the genetic background for cereulide toxin production. The level of emetic *B. cereus* in the positive samples ranged from 0.1 to 20 CFU/g of ice cream.

Zhou *et al.* (2010) reported the occurrences of *Bacillus cereus* group strains in 40 ice cream samples. Among 109 isolated *B. cereus* group strains confirmed by 16S rDNA sequence analysis only 50 were identified as *B. cereus* and one as *B. thuringiensis* by using FDA (U.S. Food and Drug Administration) standard, indicating the two identification standards were highly inconsistent. Furthermore, the psychrotolerant growth properties and the occurrence of specific psychrotolerant genes of the isolates were also studied. Both psychrotolerant 16S rDNA fragments and enterotoxic genes could be detected among mesophilic and psychrotolerant strains. No relationship among psychrotolerance, presence of

psychrotolerant 16S rDNA fragments and enterotoxic genes were found and the specific cspA fragment was only detected in a small fraction (9.5%) of the psychrotolerant isolates. One psychrotolerant isolate Bw2-1 was identified as *B. weihenstephanensis,* but no clear distinguishing characteristics between *B. weihenstephanensis* and psychrotolerant *B. cereus* were found.

Panagou *et al.* (2009) developed a product-specific model and validated under dynamic temperature conditions for predicting the growth of *Listeria monocytogenes* in pasteurized vanilla cream, a traditional milk-based product. Model performance was also compared with Growth Predictor and Sym'Previus predictive microbiology software packages. Commercially prepared vanilla cream samples were artificially inoculated with a five-strain cocktail of *L. monocytogenes,* with an initial concentration of 102 CFU g(-1), and stored at 3, 5, 10, and 15 ^{0} C for 36 days. The growth kinetic parameters at each temperature were determined by the primary model of Baranyi and Roberts. The maximum specific growth rate (mu(max)) was further modeled as a function of temperature by means of a square root-type model. The performance of the model in predicting the growth of the pathogen under dynamic temperature conditions was based on two different temperature scenarios with periodic changes from 4 to 15 ^{0} C. Growth prediction for dynamic temperature profiles was based on the square root model and the differential equations of the Baranyi and Roberts model, which were numerically integrated with respect to time. Model performance was based on the bias factor (B(f)), the accuracy factor (A(f)), the goodness-of-fit index (GoF), and the percent relative errors between observed and predicted growth. The product-specific model developed in the present study accurately predicted the growth of *L. monocytogenes* under dynamic temperature conditions.

Akalin *et al.* (2008) showed the effects of supplementation of oligofructose or inulin on the rheological characteristics and survival of *Lactobacillus acidophilus* La-5 and Bifidobacterium animalis Bb-12 in low-fat ice cream stored at -18 ^{0} C for 90 day . Addition of oligofructose or inulin to ice cream mix significantly increased apparent viscosity and overrun and developed the melting properties in ice cream during storage ($P < 0.05$). However, the highest increase in firmness, the lowest change in melting properties, and the longest 1st dripping time were obtained in

probiotic ice cream containing inulin ($P<0.05$). Some textural properties have also improved especially by the end of storage. Freezing process caused a significant decrease in the viability of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 ($P < 0.05$). Oligofructose significantly improved the viability of L. acidophilus La-5 and *B. animalis* Bb-12 in ice cream mix ($P < 0.05$). Although the viable numbers for both bacteria decreased throughout the storage, the minimum level of 10(6) CFU/g was maintained for *B. animalis* Bb-12 in only ice cream with oligofructose during storage.

Gougouli *et al.* (2008) reported the kinetic behavior of *Listeria monocytogenes* in 2 commercial ice cream products (A and B) that were inoculated and stored under static chilling (4 to 16 ^{0} C), static freezing (-5 to -33 ^{0} C), dynamic chilling, and dynamic chilling-freezing conditions simulating conditions of the aging process and of normal or abuse conditions during distribution and storage. The ice cream products A and B had different compositions but similar pH (6.50 and 6.67, respectively) and water activity (0.957 and 0.965, respectively) values. For both chilling and freezing conditions, the kinetic behavior of the pathogen was similar in the 2 products, indicating that the pH and water activity, together with temperature, were the main factors controlling growth. Under chilling conditions, *L. monocytogenes* grew well at all temperatures tested. Under freezing conditions, no significant changes in the population of the pathogen were observed throughout a 90 day storage period for either of the inoculum levels tested (10(3) and 10(6) cfu/g). Growth data from chilled storage conditions were fitted to a mathematical model, and the calculated maximum specific growth rate was modeled as a function of temperature by using a square root model.

Grossi *et al.* (2008) observed that microbial concentration, essential for safe and high quality food products, is traditionally made with the plate count technique, that is reliable, but also slow and not easily realized in the automatic form, as required for direct use in industrial machines. To this purpose, the method based on impedance measurements represents an attractive alternative since it can produce results in about 10h, instead of the 24-48h needed by standard plate counts and can be easily realized in automatic form. In this paper such a method has been experimentally studied in the case of ice-cream products. In particular, all main ice-

cream compositions of real interest have been considered and no nutrient media has been used to dilute the samples. A measurement set-up has been realized using benchtop instruments for impedance measurements on samples whose bacteria concentration was independently measured by means of standard plate counts. The obtained results clearly indicate that impedance measurement represents a feasible and reliable technique to detect total microbial concentration in ice-cream, suitable to be implemented as an embedded system for industrial machines.

Schrijver *et al.* (2008) reported an outbreak of verocytotoxin-producing *Escherichia coli* (VTEC) O145 and *E. coli* O26 occurred among consumers of ice cream produced and sold in September 2007 at a farm in the province of Antwerp (Belgium). The ice cream was consumed at two birthday parties and also eaten at the farm. Five children, aged between two and 11 years, developed haemolytic uraemic syndrome (HUS), and seven other co-exposed persons contracted severe diarrhoea. In three of the five HUS cases VTEC O145 infections were laboratory confirmed, one in association with VTEC O26. Identical isolates of E. coli O145 and O26 were detected with PCR and PFGE in faecal samples of patients and in ice cream leftovers from one of the birthday parties, in faecal samples taken from calves, and in samples of soiled straw from the farm at which the ice cream was produced.

Zhang *et al.* (2008) confirmed the contamination of *Staphylococcus aureus* isolates in ice cream by phenotypic typing and molecular typing. Two *Staphylococcus aureus* isolates were separated, one from ice cream, another from cutter. Their characteristics of drug-resistance, staphylococcal enterotoxin (SEA-E), SE (A-E,G-J) genes and PFGE type were the same.

Karahan *et al.* (2006) used a mixture of human-derived probiotic strains of *Lactobacillus acidophilus, L. agilis* and *L. rhamnosus* was used as a probiotic culture in ice cream manufacture. Viability and survival of these probiotic cultures were investigated in two different ice cream formulations. Ice cream with sucrose and ice cream with aspartame were prepared and each of these was divided into two

subgroups: one with direct addition of the probiotic culture and one with milk fermented by the same probiotic culture. Ice cream samples were stored at -20 ^{0} C for 6 months and the survival rate of cultures were determined monthly. Probiotic cultures underwent tests for resistance to bile salts, antibiotics, acidic conditions; they were found to be highly resistant to such challenges. Chemical analysis of ice cream samples, such as determination of acidity, pH and solid matter, was also performed.

Holm *et al.* (2002) reported the efficacy of a 62 h cleaning frequency in the manufacturing of ice-cream. Various product and product contact surfaces were sampled progressively throughout the time period between cleaning cycles, and analyzed for microbial growth. The coliform and standard plate counts (SPC) of these samples did not vary significantly over time after 0, 24, 48, or 62 h from Cleaning in Place (CiP). Data for product contact surfaces were significant for the SPC representing sample locations. Some of the variables in cleaning practices had significant influence on microbial loads. An increase in the number of flavors manufactured caused a decrease in SPC within the 24 h interval, but by the 48 h interval the SPC increased. More washouts within the first 24 h interval were favorable, as indicated by decreased SPC. The more frequently the liquefier was sanitized within the 62 h interval, the lower the SPC.

Kruy *et al.* (2001) reported of the microbiological quality of ice creams/sorbets sold on the streets of Phnom Penh city. Socio-demographic and environmental characteristics with two ice/ice creams samples were collected from vendors selected in the city. A total of 105 vendors and 210 ice/ice creams samples were randomly selected for the study period. Ice/ice cream vendors in the streets of Phnom Penh were adults (mean age: 28 years old) with a male predominance (86.5%). Mean educational level of vendors was 5 years with no training in mass catering. Most ice creams and sorbets (81.7%) were made using traditional methods. Microbiological analysis performed in the laboratory of Pasteur Institute of Cambodia indicated the poor bacteriological quality of the samples. The proportions of samples classified unsafe according to microbiological criteria were 83.3% for total bacterial count at 30 degrees C, 70% for total coliforms, 30% for

faecal coliforms, 12.2% for *Staphylococcus aureus* and 1.9% for presence of *Salmonella spp.*

Nissen *et al.* (2001) reported the germination and growth of both inoculated and naturally occurring *Bacillus* strains in heat-treated cream with and without nisin. Spores of several *Bacillus* species may survive heat treatment of cream, but low concentration of nisin with inhibit germination and growth.

Arias *et al.* (2000) showed the presence of total and fecal coliforms, *E. coli, Listeria. sp* and *Salmonella. sp.* was evaluated in 65 samples of both commercial and homemade ice cream. 37.1% of homemade ice cream and 20% of commercial ice cream did not fulfill the international standard for total coliforms. At the same time 82.9% of home made samples and 56.7% of commercial ones presented fecal coliforms. *E. coli* was found in 51.4% of home made samples and 26.7% of commercial ones. Sixteen *Listeria. sp.* isolates were obtained, 50% corresponded to *Listeria monocytogenes* and 50% to L. innocua. The overall presence of *L. monocytogenes* in ice cream samples was of 12.3% and it was isolated in all cases, from homemade ice cream samples.

Baylis *et al.* (2000) compared the ability of four rapid methods and a standard cultural method to detect low levels of heat-injured cells of *Salmonella typhimurium* in ice cream and skimmed milk powder. The detection of Salmonella in samples contaminated with low levels (< 10 cfu 25 g^{-1}) was significantly greater with the novel broth method than with the other methods (P </= 0.01). At contamination levels > 10 cfu 25 g^{-1}, there was no significant difference between the methods except for the novel broth method and a dipstick-based immunoassay (P </= 0.05). The novel broth method, S.P.R.I.N.T. Salmonella, which incorporates a specifically formulated peptone and Oxyrase(R) combination followed by the timed release of selective agents into the recovery medium, was shown to improve the rate of detection of low numbers of injured cells of *Salmonella* after 24 h enrichment.

Kamat *et al.* (2000) examined the efficacy of low-dose irradiation to improve the microbial safety of ice cream. Initially three different flavors (vanilla, strawberry and chocolate) of ice cream were exposed, at -72 ^{0} C, to doses of 1, 2, 5, 10 and 30 kGy to gamma-radiation. Irradiation at 1 kGy resulted in reduction of microbial population by one log cycle, thus meeting the requirement limits prescribed by Bureau of Indian Standards. Pathogens such as *Listeria monocytogenes* 036, *Yersinia enterocoliticta 5692* and *Escherichia coli* O157:H19, respectively, showed the D10 values 0.38, 0.15 and 0.2 kGy in ice cream at -72 ^{0} C suggesting the efficacy of low doses (1 kGy) in eliminating them. Sensory evaluation studies of ice cream irradiated at 1, 2, 3 and 5 kGy by a 15 member panel demonstrated that doses higher than 2 kGy irradiation induced off-odour and an aftertaste was evident in vanilla ice cream. A radiation dose of 1 kGy was sufficient to eliminate the natural number of pathogens present in the ice cream. No statistically significant differences were observed in the sensory attributes of all the three flavours of ice cream either unirradiated or exposed to 1 kGy ($P < 0.05$).

Ranaivo *et al.* (2000) evaluated the microbiological quality of street-vended ice creams in Dakar. A total of 313 samples of ice creams from 170 street-vendors were collected and tested for common foodborne pathogens and indicator organisms. Results showed that microbiological quality of 45% of tested samples was unsatisfactory because of large populations of aerobic mesophilic organisms (36.7%), thermotolerant coliforms bacteria (21.4%) and sometimes *E. coli.* (10.6%). Strict pathogens as *Salmonella, Shigella* and *Vibrio cholerae* were not found.

Aboshkiwa *et al.* (1999) analysed Seventy (70) appendiceal specimens and 80 ice-cream samples to detect *Yersinia enterocolitica* using three different media. Both *Y. enterocolitica* and *Citrobacter freundii* were recovered in appendiceal specimens (17.1% and 8.6%) and ice-cream (26.25% and 18.75%) respectively. Thioglycollate medium was more selective and productive in isolating *Yersinia. Y. enterocolitica* was the major causative agent of acute appendicitis (44%).

Miettinen *et al.* (1999) found one dominating strain of serotype 1/2b when serotyping and pulsed-field gel electrophoresis (PFGE) patterns were used for the characterization of 41 *Listeria monocytogenes* isolates originating from an ice cream plant. Samples were taken from the production environment, equipment and ice cream during the years 1990-1997. Serotyping divided the isolates into two serovars, 1/2b and 4b. Three rare-cutting enzymes (ApaI, AscI and SmaI) were used in the creation of PFGE patterns. AscI resulted in the best restriction enzyme digestion patterns (REDPs) for visual comparison. Eight different AscI REDPs were obtained, whereas ApaI produced six and SmaI seven banding patterns. When one-band differences are taken into account, 12 different PFGE types were distinguished based on information obtained with all three enzymes. The dominant PFGE type was found to have persisted in the ice cream plant for seven years.

Dodhia *et al.* (1998) reported an outbreak of food poisoning caused by *Salmonella enteritidis* phage type (PT) 6 followed a children's birthday party. Thirty of 37 (attack rate 81%) attendees were ill, with diarrhoea and fever being the most common symptoms. One child required hospital admission for rehydration. No deaths occurred. Stool specimens from 24 out of 25 people who submitted them were positive for *S. enteritidis* PT 6 and one was positive for *S. enteritidis* PT4. Epidemiological investigation indicated that home-made ice cream was the vehicle of infection. Fresh shell eggs used raw in the ice cream were the likeliest source of infection. No ice cream or eggs were available for microbiological analysis. *S. enteritidis* PT6 accounts for a small but increasing proportion of all salmonellas in humans and its epidemiology is thought to be similar to *S. enteritidis* PT4.

Tawila *et al.* (1998) used the HACCP system to study the preventive food safety approach to control some hazards appearing in one of the ice cream production plants in Egypt. The problem comprised the presence of bacteriological and some chemical contamination in most of the company products. Before applying the HACCP system, the samples examination showed high total mesophilic plat count in 50% of the samples and high coliform count in all samples compared to the level recommended in the Egyptian standards. The highest *staphylococcal* count (negative for coagulase test) obtained was that of chocolate (1.3

x 10^4 CFU/g) followed by mango ice cream (1.0 x 10^4 CFU/g). Faecal coliform was only positive in mango ice cream.

Vought *et al.* (1998) examined nine ice cream sample containers, representing three production lots involved in a 1994 outbreak of salmonellosis, were obtained from the manufacturer's distribution warehouse and from consumers. Single 100g samples from each container were tested initially, with analyses beginning seven weeks after the ice cream was produced. Quantitative salmonella analysis of these samples was performed 14 weeks after the production date using a three-replicate, three-dilution most probable number (MPN) procedure using 100-, 10-, and 1-g samples. The MPN/g estimates ranged from 0.004 to 0.46 *Salmonella enteritidis* per g with six of nine samples showing an MPN value of 0.093 *S. enteritidis* per g. The 95% confidence interval for *S. enteritidis* among these samples was < 0.001 to 2.4 cells/g. The 95% upper limit of *S. enteritidis* per g was 0.38 for five of six consumer samples.

Gooding *et al.* (1997) detected *Escherichia coli* O157:H7 in spiked samples of raw milk and ice-cream by immunomagnetic separation, subjected to amplification by polymerase chain reaction of parts of the verotoxin genes (SLT-I and SLT-II), and detected by agarose gel electrophoresis. Using this method, as few as 1 cfu. *E. coli* O157:H7/g food could be detected in < 10 h.

Hammack *et al.* (1997) determined the relative efficacies of hemorrhagic coli (HC) agar and several formulations of sorbitol MacConkey (SorMac) agar, with and without 0.1% (w/v) 4-methyllumbelliferyl-beta-D-glucuronide (MUG), in recovering unstressed and heat-stressed *Escherichia coli* O157:H7 from Brie cheese, ice cream, and whole milk . Recovery of unstressed *E. coli* O157:H7 was determined quantitatively by spread-plating diluted samples onto different agars and performing plate counts. Recovery of stressed *E. coli* O157:H7 was determined qualitatively by enriching samples in modified trypticase soy broth, streaking the incubated enrichments, and isolating *E. coli* O157:H7 colonies from the agars. HC agar and the SorMac agar formulations did not differ significantly in their ability to recover unstressed *E. coli* O157:H7 from ice cream and whole milk; however, HC

agar recovered significantly more unstressed *E. coli* O157:H7 from Brie cheese than did the SorMac agar formulations.

Wilson *et al.* (1997) determined the effect of scoop water hygiene on the microbiological quality of ice cream. An aerobic plate count around 10^6 c.f.u/ml was the modal value for scoop waters. Unopened ice creams generally had counts around 10^3-10^4 c.f.u/ml and this increased by one order of magnitude when in use. Many scoop waters had low coliform counts, but almost half contained > 100 c.f.u/ml. E. coli was isolated in 18% of ice creams in use, and in 10% of unopened ice creams. *S. aureus* was not detected in any sample.

Wouafo *et al.* (1996) showed the hygienic quality of ice cream produced in two main towns in Cameroon (Douala and Yaoundé) for their hygienic quality. The microbiologic examinations show that many of them were contaminated with bacteria of faecal origin, pathogenic *Staphylococcus* and *Salmonella* respectively in 71.3, 49.6 and 5% of the products examinations in the study. The use of non potable water and the disrespect of the hygienic rules during the production were the main causes of contamination. Recommendations were made to preserve the public health in the developing country.

Morgan *et al.* (1994) reported an outbreak of *Salmonella enteritidis* PT4 infection in which home-made ice cream was identified as the vehicle of infection. The ice cream contained approximately 10^5 cfu/ml *S. enteritidis* PT4 organisms per gm and was probably contaminated by an infected shell egg containing between 10^5-10^8 cfu/ml organisms. The continued relevance of the Chief Medical Officer's warning on the use of raw shell eggs is highlighted. Home-made ice cream using the same recipe as ice cream that had been incriminated as the cause of the family outbreak of *S. enteritidis* PT4 infection was used to study the growth of the organism that might have occurred in the 3-4 h it took to prepare the product. When the inoculum was in the stationary phase, as it would be from shell or other cross contamination, there was a lag phase of 3 h before growth occurred at room temperature.

Maifreni *et* al. (1993) analysed artisanal ice-cream from different ice-cream shops in Udine and province and analysed. All tested flavours contained aerobic germs, coliforms, *Enterococci* and yeasts in different quantities. In the analysed samples neither *Salmonellae spp.*, nor *Listeria monocytogenes* nor *Staphylococcus aureus* were detected. Numerous species of coliforms and yeasts were randomly isolated and identified. The statistical analysis, used to compare the variables (flavour, month, year), showed significant differences among the samples analysed in the two years. In September, the means of the total aerobic count were significantly different from the ones of mediums, are better than those ones, which use only selective mediums. Lastly, with July and of August. No significant differences were noted in the means of coliforms, total aerobic counts and yeasts in the different flavours.

McMahon *et al.* (1992) made probiotic ice cream by fermenting a standard ice cream mix with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cultures and then freezing the mix in a batch freezer. Survival of the *L. acidophilus* and *B. bifidum,* as well as beta-galactosidase activity, was monitored during 17 wk of frozen storage at -29 ^{0} C. After freezing of the fermented mix, bacterial counts were 1.5 x 10^{8} cfu/ml for *L. acidophilus* and 2.5 x 10^{8} cfu/ml for B. bifidum. Seventeen weeks after freezing, these counts had decreased to 4 x 10^{6} and 1 x 10^{7}cfu/ml, respectively. During the same period, beta-galactosidase activity decreased from 1800 to 1300 units/ml. Probiotic ice cream was prepared at pH 5.0, 5.5, and 6.0 to determine consumer preferences and was compared with standard Utah State University "Aggie" ice cream.

García *et al.* (1989) examined a total of 122 samples of vanilla ice cream, the base product used for all flavors, prepared by eight different large firms at the Metropolitan Area of Caracas, Venezuela, were analyzed for aerobic mesophilic and psicrophilic bacteria, *Staphylococcus aureus,* Enterobacteriaceae, and Filamentous fungi. Findings revealed that within the sampling, 56.6% complied with the international standards proposed for aerobic mesophilic bacteria, 68% for *Staphylococcus aureus,* and 23% for Enterobacteriaceae. Three serotypes of enteropathogenic *Escherichia coli,* one of *Salmonella,* and one of *Shigella* were found. Ten genera of Filamentous fungi were isolated and identified.

Guzmán *et al.* (1989) reported the sanitary quality of ice-creams and the presence of pathogenic or potentially pathogenic species of Salmonella and *Yersinia enterocolitica.* They examined 50 samples from 5 different industrial and semi-industrial producers in San Luis (Argentine). The enumeration of coliforms was positive for all the samples with values less than or equal to 20/g. Fourteen per cent of the samples were positive for the investigation of *Staphylococcus aureus* in 1 g. For the plates enumeration 12.0% of the samples gave less than 10 u.f.c./g, 4.0% between 101 and 1000 and 4.0% between 1001 and 10,000. Fifteen strains were isolated, 26.6% biotype A (human ecovar) and the others biotype C (bovine ecovar). All of them were susceptible to chloramphenicol, cephalosporin and erythromycin; 46.6% to penicillin G and ampicillin; 93.3% to kanamycin (6.6% intermediate ones = I); 73.3% to methicillin (26.6% I); 86.6% to tetracycline (13.3% I). Six per cent of the samples over came the acceptability limit for *S. aureus. Salmonella spp* was not isolated. In 4.0% of the samples *Y. enterocolitica* were isolated, one of them typified as B1; 0:3, 50, 51, Lis Xz. The latter, isolated in samples with values of coliforms inferior to the limit fixed by some legislations, suggests a post elaboration contamination.

Masud *et al.* (1989) examined fifty samples of ice-cream. Of these 72% had total viable count over 10^6/g while 66% had coliform count between 10^2-10^3/g. The micro-organisms isolated were *Escherichia coli* (46%), *Enterobacter aerogenes* (34%), *Staphylococcus aureus (26%), proteus species* (16%), *Streptococcus faecalis* (12%), *Citrobacter species* (10%) and *Bacillus cereus* (4%).

Slavchev *et al.* (1986) reported the development and survival of *Yersinia enterocolitica* organisms in pasteurized milk and ice-cream that had been contaminated with varying amounts of microbial cells. The milk was kept at 4 degrees, 9 degrees, and 21 degrees C for 120 days, and the ice-cream at--18 degrees and --23 degrees C for 8 months. *Yersinia* development and survival were found to be dependent on temperature as well as on the numbers and activity of microflora. When milk was kept at temperatures below 10 degrees C the organisms retained viability for more than 120 days, while at room temperature (20 degrees--22 degrees C) they remained active for up to 30-60 days. The presence of coliform bacteria

inhibited the development of Yersinia, its replication and survival rates being reduced.

Palma *et al.* (1984) evaluated a total of 70 samples of ice-creams, milk, milk cream and water or fruit based from 7 different industrial producers. Of these samples, 14.3% were positive for *Staphylococcus aureus* in 1 g: 97.2% showed less than 10 *St. aureus* in 1 g, 1.4% between 101-1,000 and 1.4% between 1,001-10,000. In 21 strains enterotoxigenicity, was investigated, as well as the response to different biochemical tests and the susceptibility to antibiotics: 9.5% of the strains were toxigenic (2 strains), 1 produced enterotoxin A and 1 enterotoxin B. There was no difference between toxigenic and nontoxigenic strains when the biochemical tests were performed (catalase test, coagulase test, thermostable nuclease production, anaerobic utilization of glucose and mannitol, gelatin and casein hydrolysis, production of hemolysins and pigments).

Slavchev *et al.* (1984) reported the cold resistance of enterotoxinogenic strains of *Staphylococcus aureus* and of A and C2 *Staphylococcus enterotoxins* in milk and cream ice cream kept under refrigerator conditions for 7 months at -18 degrees C. The *S. aureus* strains were shown to have high resistance in ice cream. The survival rate of the organisms depended both on the specific peculiarities of the individual strains and on the composition of the ice cream mixture. *Staphylococci* were found to survive longer in milk icecream. A and C2 enterotoxin retained fully their activity in the two types of ice cream over the entire period of storing.

Aleksieva *et al.* (1983) examined a total of 86 batches of Eskimo ice-cream and 101 batches of 'cream' ice-cream. It was found that 83.6 per cent of the Eskimo batches were of 0.1 coliform titers and higher, and 16.3 per cent - of 0.01 coliform titers. The total microbial contamination of 86 per cent of the batches reached 50000 g, while that of 13.9 per cent reached 50000 to 180000/g. The colititer with 98 per cent of the batches of 'cream' ice-cream was 0.1 and more 0.1, and with 1.98 per cent only was it 0.01, while the microbial count with 96 per cent of these batches was up to 50000/g, and with 3.9 per cent it was from 50000 to 118000/g. The total microbial

content of the surface layer varied from 200 to 46000/g, dropping up to 100-2100/g at storage.

Köşker *et al.* (1981) isolated coagulase-positive *Staphylococci* from different kinds of cheese and ice-cream sold in Ankara and some biochemical properties of these isolates were determined. A total of 55 cheese, 52 ice-cream (107 samples) were examined for the presence of coagulase-positive *Staphylococci*. Baird Parker Medium was used and 26 samples constituting of 13 cheese and 13 ice-cream were found to be contaminated with coagulase-positive *Staphylococci* and ratio of the contaminated samples to the total was calculated as 24.3%. Highest count was determined to be 176, 166/g in Izmir Tulum Cheese, whereas none of the other tulum cheese samples yielded this bacteria. In general, coagulase-positive *Staphylococci* of cheese samples were higher than ice-cream samples. Among the ice-cream samples highest coagulase-positive *Staphylococci* count was obtained in nutty ice-cream. From 26 contaminated samples 164 coagulase-positive *Staphylococci* were isolated.

Markakis *et al.* (1978) reported 22 outbreaks of salmonellosis associated with the consumption of homemade ice cream. *Salmonella typhimurium* accounted for 45% of the outbreaks. The source of eggs used was known in 13 outbreaks, and all were ungraded farm- or home-produced eggs, a potential source of salmonellae. In 11 outbreaks, the method of preparation was known, and in all, the ice-cream custard had not been cooked before freezing.

Aleksie *et al.* (1977) reported that the presence of *Enterococci* was reduced up to 21 per cent of the samples only had from 101 to 2000 microbial cells per cu. cm of the ice-cream mass. *Enterococci* of the fecal group predominated 81 per cent. The regime of thermal treatment applied to the ice-cream melange produced bactericidal effect on the *Enterococcus* microflora. After pasteurization, however, the product became contaminated with a secondary Enterococcus infection due to the improperly cleaned glassware and equipment. In freezing the ice-cream melange the amount of Enterococcus microflora dropped from 3.2 to 3.3 times. No changes in the microbial count were established after the deep-freezing storage of ice-cream for 24 months at -18 to -25 degrees C.

Kietzmann *et al.* (1975) explained two important hygienic-bacteriological issues: 1. Which is the capacity of survival owned by microorganisms in icecream in correspondence to temperature and storing time, and what significance have the results in regard to the length of intervals between the taking of samples of icecream and their examination. 2. Which nutritient media are suitable for a routine diagnostic of coliforms. Analyses were being made with an artificially contaminated, industrially produced icecream (Vanilla icecream with 10 per cent milk fat and strawberry icecream with 6,6 per cent milk fat and 21 per cent strawberries). Inoculations were only done with strains of bacteria isolated from icecream. They employed one strain of *Escherichia coli, Staphyloccus aureus, Pseudomonas aeruginosa* and three strains of Coliforms for each inoculation. Contaminated samples were stored in deep-freeze units at three different temperatures (-28, -18, -8 degrees C). In the beginning inspections in regard to microbial content were performed in daily intervals, but intervals grew longer in the course of time. At the utmost samples were under observation for 140 days. For quantitative measurement of Coli and Coliform microorganisms five different culture media were used (Endoagar, Hexachlorophene Endoagar, Desoxycholatcitrat Agar, Violet Red Bile Agar and Brilliant Green Broth). They showed that icecream, if being stored, looses approximately one to two quarters of the bacteria within hours after production, the third dies within days and the rest after weeks and months.

Chapter 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The present research work was conducted during July to December, 2012 in the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The detailed outline of Materials and Methods are given below.

3.1 Materials

3.1.1 Study area

Ice cream samples were collected from the local market from the Dinajpur district of Bangladesh. The samples were collected and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis.

3.1.2 Laboratory preparation

All items of glasswares including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides, vials and agglutination test tubes soaked in a household dishwashing detergent solution ('Trix, Recket and Colman Bangladesh Ltd.) for overnight, contaminated glasswares were disinfected in 2% sodium hypochloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized either by dry heat at 160^0 C for 2 hours or by autoclaving for 15 minutes at 121^0 C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven over at 50^0 C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50^0 C for future use.

3.1.3 Instrument and appliances

Phase contrast microscope, digital pH meter, test tubes, cotton, hand gloves, plastic syringe (5 ml), micropipette (1 ml, 500 µl, 10-20 µl), glass slides, eppendorf tubes, magnifying glass, marker pen, ice-box, spirit lamp, cover slips, inoculating loop and rack, autoclave, refrigerator, conical flask, etc.

3.1.4 Media for culture

3.1.4.1 Liquid media

- Nutrient broth.
- Lactose broth (Hi-media, India).
- Peptone broth (Hi-media, India).
- 1% peptone water (Hi-media, India).

3.1.4.2 Solid media

- Nutrient agar base (Hi-media, India).
- Plate count agar media (Hi-media, India).
- Staphylococcus agar no. 110 (Hi-media, India).
- Eosin methylene blue (EMB) agar (Hi-media, India).
- MacConkey agar medium (Hi-media, India).
- Triple sugar iron (TSI) agar slant (Hi-media, India).
- Motility, Indole, Urease (MIU) medium (Hi-media, India).

3.1.5 Reagents

- Gram's staining reagent: Crystal violet, Gram's iodine, Acetone and Safranine.
- Alpha-naphthol solution.
- Kovac's reagent.
- Ethyl alcohol (70% and 95%).
- Sugar media (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) and other chemicals and reagents.

3.1.4.1 Liquid Media

3.1.4.1.1 Nutrient broth

Nutrient broth (NB) was used to grow the organisms from the samples collected from the study areas before performing biochemical test (Cheesebrough , 1984).

3.1.4.2 Solid media

3.1.4.2.1 Plate count agar

Plate count agar was used to determine of plate count of microorganisms by pour plate method (Cheesebrough , 1984).

3.1.4.2.2 Nutrient agar

Nutrient agar (NA) medium was used to grow the organisms from the collected samples (Cheesebrough , 1984).

3.1.4.2.3 Staphylococcus agar no. 110

Staphylococcus agar no. 110 was used as a selective medium for *Staphylococci* which causes enhancement of the growth of *Staphylococcus* while inhibiting the growth of other contaminating organisms and shows typical colony characters (Cheesebrough , 1984).

3.14.24 MacConkey agar

MacConkey agar (MC) medium was used to culture the organisms under the family Enterobacteriaceae (Cheesebrough , 1984).

3.1.4.2.5 Eosin methylene blue agar

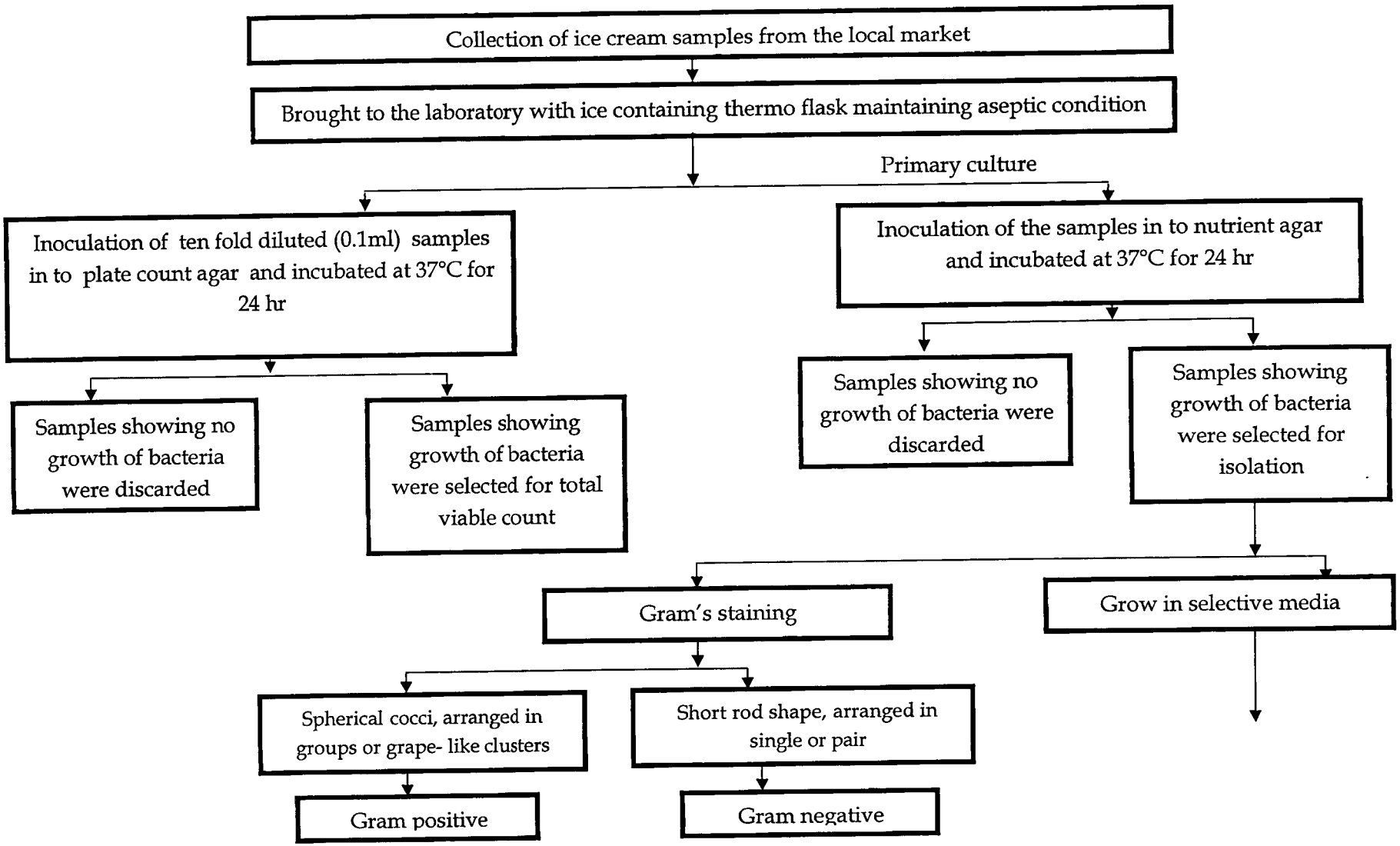
Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesebrough , 1984).

3.2 Methods

3.2.1 Experimental layout

The ice cream samples were collected directly from local market of Dinajpur town for the isolation and identification of bacterial pathogens by morphology, staining and cultural characteristics. Characterization of bacteria were done by cultural and biochemical reactions.

EXPERIMENTAL LAYOUT



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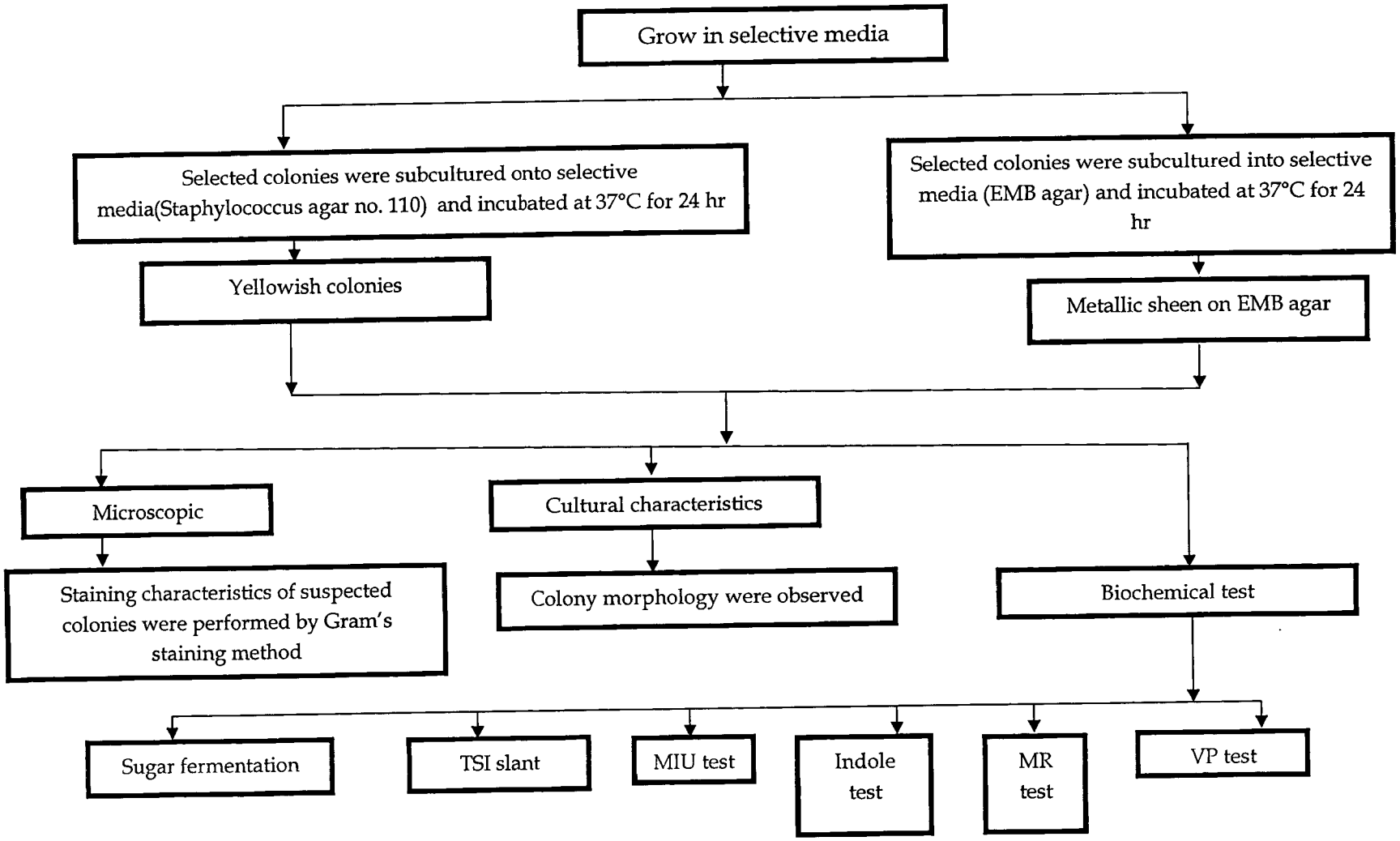

Grow in selective media
Selected colonies were subcultured onto selective media(Staphylococcus agar no. 110) and incubated at 37°C for 24 hr
Selected colonies were subcultured into selective media (EMB agar) and incubated at 37°C for 24 hr
Yellowish colonies
Metallic sheen on EMB agar
Microscopic
Cultural characteristics
Biochemical test
Staining characteristics of suspected colonies were performed by Gram's staining method
Colony morphology were observed
Sugar fermentation
TSI slant
MIU test
Indole test
MR test
VP test

3.2.2 Collection of ice cream sample

Ice cream samples from local market were collected aseptically and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination.

3.2.3. Enumeration of total viable count (TVC)

For the determination of total bacterial count, 0.1 ml of each ten-fold dilution was transferred and spread on dupli-cate plate count agar using a fresh pipette for each dilution. The diluted samples were spread as quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate.

The plates were then kept in an incubator at 30°C for 24-48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total viable count. The total viable count was calculated according to ISO. The results of the total bacterial count were expressed as the number of organism or colony forming units per gram (CFU/gm) of ice cream sample.

3.2.4. Enumeration of total *E. coli* count (TEC)

For the determination of total *E.coli* count 1ml of each tenfold dilution was transferred to EMB agar. For each dilution five test plates containing EMB agar were used. All the agar plates were incubated at 30°C tem-perature for 48 hours.

The total coliform count was calculated according to ISO. The results of the total coliform count were expressed as the number of organism or colony forming units per gram (CFU/gm) of ice cream sample.

3.2.5. Enumeration of total *Staphylococcal* Count (TSC)

For the determination of total Staphylococcal count 1ml of each tenfold dilution was transferred to staphylococcus agar no.110 agar. For each dilution five test plate containing Staphylococcus agar no.110 agar were used. All the agar plates were

incubated at 37°C tem-perature for 48 hours. The total staphylococcal count was

calculated according to ISO. The results of the total Staphylococcal count were expressed as the number of organism or colony forming units per gram (CFU/gm) of ice cream sample. A total of 3 samples of each brand was bacteriologically examined. The results were recorded and the organoleptic and microbiological quality was determined.

3.2.6 Isolation and identification of pathogens

The entire ice cream samples were selected for bacteriological culture.

3.2.6.1 Preparation of culture media and reagents

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.2.6.1.1 Liquid Media:

3.2.6.1.1.1 Nutrient broth

Thirteen grams of Bactonutrient broth (Difco) was dissolved in 1000 ml of cold distilled water and heated upto boiling to dissolve it completely. The solution was then distributed in tubes, stoppered with cotton plugs and sterilized in the autoclave machine at 121°C and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was judged by incubating overnight at 37°C and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.6.1.1.2 Sugar media

The medium consists of peptone water of which fermentable sugar added to the proportion of 100%. One gram of Bacto peptone (Hi-media, India) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled

for 5 min, adjusted to pH 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation and placed inverted. These were then sterilized by autoclaving at 15 lb/sq.inch 121^{0}C for 15 minutes. The sugar used for fermentation was prepared separately 10% solution in distilled water (10 gram sugar dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar solutions were sterilized in Arnold steam sterilizer at 100^{0}C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at for 24 hours (Carter, 1979).

3.2.6.1.2 Solid media

3.2.6.1.2.1 Plate count agar

Seventeen grams of plate count agar powder was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^{0}C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.3.1.2.2 Nutrient agar

Twenty eight grams of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^{0}C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.6.1.2.3 Staphylococcus agar no. 110

One hundred grams of Staphylococcus agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. The precipitate was resuspended by gentle agitation to avoid bubbles and pour the plates while the medium is hot and allowed to solidify. Alternatively, the medium

was cooled to 45-50^{0}C and blood or egg yolk was added if desired. Staphylococcus agar no. 110 may also be used without sterilization; it should be boiled for 5 mins used at once. After solidification of the medium in the petridishes, these were incubated at 37^{0}C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.6.1.2.4 Eosin methylene blue agar

Thirty six grams of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed.

The sterility of the medium was judged and used or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.6.1.2.5 MacConkey agar

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.6.1.2.6 MIU medium

Eighteen grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. Ninety five ml was despensed into flasks and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then was Cooled to about 50-55°C and aseptically add 5ml was added of sterile 40% basal medium. After mixing were dispensed into sterile test tubes. Allow to cool in an upright position. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.6.1.3 Reagents preparation

3.2.6.1.3.1 Methyl Red-Voges Proskaure test

3.2.6.1.3.1.1 Methyl Red-Voges Proskaure broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1984).

3.2.6.1.3.1.2 Methyl red solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water.

3.2.6.1.3.1.3 Voges-Proskauer solution

3.2.6.1.3.1.3.1 Alpha-naphthol solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol.

3.2.6.1.3.1.3.2 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in 100 ml of cold distilled water.

3.2.6.1.3.2 Indole test

3.2.6.1.3.2.1 Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of amyl alcohol and to this mixture 5 grams of paradimethyl-amino-henzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1967).

3.2.6.1.3.3 Phosphate buffered saline solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride (NaCl), 2.89 gram of disodium hydrogen phosphate ($Na_2HPO_4, 12H_2O$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate

were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121 °C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1984).

3.2.6.2 Culture of ice cream sample

Each sample of ice cream earlier put into transport media was divided and inoculated separately in nutrient agar (NA) and plate count agar to promote growth of bacteria. Each group of these media was incubated at 37^{0}C for overnight. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1984) until the pure culture with homogenous colonies were obtained.

3.2.6.3 Morphological characterization of organisms by Gram's staining method

- A loopful of sterile distilled water was placed in the center of a clean sterile slide.
- A Small colony was picked up with a bacteriological loop and was mixed with distilled water on the slide.
- The colony was made to thin smear on a slide.
- The smears were fixed by air drying.
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Gram 's iodine was then added to act as mordant for one minute.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was washed with water.
- Safranine was added as counter stain and allowed for one minute.
- The slide was then washed with water.
- Then the slide was blotted with blot paper and was allowed to air dry. The slide was examined under microscope with high power objective (100X) using immersion oil.

3.2.6.4 Biochemical tests

3.2.6.4.1 Sugar fermentation test

For sugar fermentation test the containing different sugar media such as sucrose, maltose, dextrose, lactose and mannitol were inoculated with a loopful of broth culture of the isolated organism and incubated at 37^{0}C for 18 hours.

3.2.6.5 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per:

Step-1: An inoculum was picked up with a sterile loop and spread on a area of the medium in the petridish.

Step-2: The loop was sterilized by being heated as red hot in a flame.

Step-3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.2.6.6 Techniques used for the isolation and identification of *Escherichia coli.*

3.2.6.6.1 Culture into different media

Sterilized platinum loop was used for streaking the lactose broth culture on MacConkey and EMB agar to get isolates in pure culture. All inoculated media were kept at 37°C for overnight in an incubator.

3.2.6.6.1.1 MacConkey agar

Materials from lactose fermentation tubes were inoculated into MacConkey agar plates and were incubated.

3.2.6.6.1.2 Eosin Methylene Blue (EMB) agar

Materials from lactose fermentation tubes were inoculated into EMB agar plates which after incubation, showed metallic sheen if positive for *Escherichia coli.*

3.2.6.6.2 Microscopic study for identification of *Escherichia coli* suspected colonies by Gram's staining

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram staining reaction was performed according to the methods described by Merchant and Packer (1967).

3.2.6.6.3 Identification of *Escherichia coli* isolates by biochemical test

3.2.6.6.3.1 Sugar fermentation test

Sugar fermentation test was performed to identify of *Escherichia coli*. For sugar fermentation on the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose and mannitol were inoculated with a loopful of broth culture of the isolated and incubated at 37° C for 18 hours.

3.2.6.6.3.2 Indole test

Two ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added and mixed thoroughly. The tube was then allowed to stand for a while.

3.2.6.6.3.3 Triple sugar iron (TSI) agar slant

The test organisms were culture into TSI agar slant by stab streak method.

3.2.6.6.3.4 Motility, Indole, Urease (MIU) test

Suspected colony was inoculated into the tube containing MIU medium. Then the medium was incubated at 37°C for overnight.

3.2.6.6.3.5 Methyl red test

The test was conducted by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate peptone broth. After 48 hours incubation at 37°C, a drop of methyl red solution was added. A red coloration is positive and indicates an acid pH of 4.5 or less resulting from the fermentation of glucose (Cheesbrough, 1984).

3.2.6.6.3.6 Voges-Proskaure test

2 ml of sterile glucose phosphate peptone water were inoculated with the 5ml of test organisms. It was incubated at 37°C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed 3 ml of α-naphthol were added and snaked well. The bottle cap was removed and

left for an hour at room temperature.

3.2.6.7 Techniques for isolation and identification of *Staphylococcus* spp.

3.2.6.7.1 Culture into different media

Loopful aliquot was taken from the nutrient broth culture and streaked on nutrient agar media to get in pure culture. All inoculated media were kept at 37^{0}C for overnight in an incubator.

3.2.6.7.1.1 Nutrient agar

Materials from nutrient broth tubes were inoculated into nutrient agar plates.

3.2.6.7.1.2 Staphylococcus agar no. 110

Materials from nutrient broth tubes were inoculated into Staphylococcus agar no 110 containing plates.

3.2.6.7.2 Microscopic study for identification of *Staphylococcus* spp. suspected colonies by Gram's staining

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram's staining reaction was performed according to the methods described by Merchant and Packer (1967). Stained slides were examined under light microscope at 100 x magnification.

3.2.6.7.3 Identification of *Staphylococcus* spp. isolates by biochemical test

3.2.3.7.3.1 Sugar fermentation test

Sugar fermentation was performed to identify of *Staphylococcus* spp. For sugar fermentation test the containing different sugar media such as sucrose, maltose, dextrose, lactose and mannitol were inoculated with a loopful of broth culture of the isolated organism and incubated at 37^{0}C for 18 hours.

3.2.6.7.3.2 Triple sugar iron (TSI) agar slant

The test organisms were culture into TSI agar slant by stab streak method and incubated at 37^0 C.

3.2.6.7.3.3 Indole test

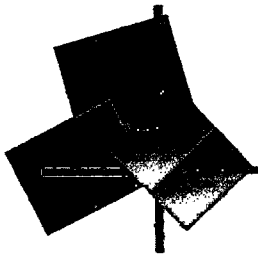
Two ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added and mixed thoroughly. The tube was then allowed to stand for a while.

3.2.6.7.3.4 Methyl red test

The test was conducted by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate peptone broth. After 48 hours incubation at 37°C, a drop of methyl red solution was added.

3.2.6.7.3.5 Voges-Proskaure test

Two ml of sterile glucose phosphate peptone water were inoculated with the 5 ml of test organisms. It was incubated at 37°C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed. Three ml of α-naphthol were added and snaked well. The bottle cap was removed and left for an hour at room temperature.



Chapter 4

RESULTS

CHAPTER 4

RESULTS

4.1 QUALITY AND TOTAL VIABLE COUNT

A number of nine commercially produced ice cream samples belonging to three different brands were collected from different retail stores located at Dinajpur town. All the samples were transported in an ice box and kept in the deep freezer of the laboratory until use. There after, the sen-sory and microbiological attribute were analyzed and studied comparatively. Sensory characteristics were judged by a panel of experts and judgement for consumer's acceptance are scored.

A presentation of the data showing the scores of characteristics of different ice cream samples are shown in Table 1. Sensory characteristics of hedonic scales obtaining scores 19-20 indicate excellent quality and 16-18 indicate good quality, 12-15 means fair; 9-11 scores recommend marginal acceptable; 7-8 considered unacceptable; 0-6 stands for bad . Five organoleptic quality characteristics were taken for the panel score and judgement for consumer's acceptance. For each of the characteristic, the maximum score given was 20 points. The grand total scores for the five characteristic parameters together become 100 points. Out of total score points 100, three different brand of ice cream samples were found to have obtained for Igloo 90 (90%) followed by Polar 68 (68%) and Kwality 57 (57%) scores. The highest score of 90 was obtained by Igloo and the low score of 57 stands for Kwality ice cream.. It is evident from the overall organoleptic quality result that ice cream samples of Igloo possessed the most superior quality, and has been liked by most of the taste panel exports. Ice cream samples were examined bacteriologically to investigate the presence of total viable microorganisms and thereby assess the sanitary quality.

The result presented in Table 2 showed the total viable bacterial load of nine different ice cream samples of three different brands. The bacterial loads were not uniform and varied quite considerably. The average counts/ml of Igloo, Polar,and Kwality were 1.19×10^4 CFU/ml (log 4.1), 1.39×10^4 CFU/ml (log 4.1) and 8.53×10^3 CFU/ml (log 3.9) respectively .The maximum and minimum range of total bacterial load per ml of ice cream samples belonging to Igloo, Polar, and Kwality varied from log 4.2 to log 4.0, log 4.2 to log 4.0, log 4.0 to log 3.8, respectively (table 3). The average total viable counts of all the three brands of ice cream was 1.14×10^4 CFU/ml (log 4.0) (table 2).

The assessment of total viable count as revealed in Table 2 has clearly provided information that the ice cream samples of Kwality and Igloo were of the superior quality, because the counts were less than the recommended microbiological standard of Food and Drug administration and USPHS. All brands of ice cream samples were within acceptable limit of public health safety because the samples did not exceed the total viable count (1, 00,000 CFU/ml) permitted under regulation.

Table 1.
Sensory characteristics of commercially produced ice cream and taste panel scores expressed in hedonic scales.

Parameters (Sensory Characteristic)		Sources of Ice Cream Samples		
		Igloo	Polar	Kwality
Appearance	20 Marks	19	13	14
Color	20 Marks	18	17	12
Odor	20 Marks	17	11	11
Texture	20 Marks	18	15	10
Overall Characteristics	20 Marks	18	12	11
Grand Total Score (100 Marks)		90	68	57

Table 2.
Density of average total viable bacteria and other selected microbial groups in per gram of ice cream samples of different brands.

Sources of ice cream samples	No. of samples analyzed	Total viable count/ml		*E. coli* count/ml		*Staphylococcal* count/ml	
		CFU	Log	CFU	Log	CFU	Log
Igloo	3	1.19×10^4	4.1	9.26×10^3	4.0	2.60×10^3	3.4
Polar	3	1.39×10^4	4.1	1.14×10^3	3.0	0.0	0.0
Kwality	3	8.53×10^3	3.9	7.95×10^2	2.9	0.0	0.0

Table 3.
Range of total viable bacterial concentration in commercial ice cream samples.

Brand specification	No. of samples analyzed	Range of TVC/ml		Average TVC/ml (mean value)	Standard deviation
		Maximum	Minimum		
Igloo	3	4.2	4.0	4.1	0.1
Polar	3	4.2	4.0	4.1	0.1
Kwality	3	4.0	3.8	3.9	0.1

All counts are expressed in logarithms

Table 4.
Range of *E. coli* counts in commercial ice cream samples.

Brand specification	No. of samples analyzed	Range of *E. coli* count/ml		Average *E. coli* count/ml (mean value)	Standard deviation
		Maximum	Minimum		
Igloo	3	4.1	3.9	4.0	0.1
Polar	3	3.2	2.8	3.0	0.2
Kwality	3	2.9	2.8	2.9	0.05

All counts are expressed are logarithms

Table 5.
Range of Staphylococcal counts in commercial ice cream samples.

Brand specification	No. of samples analyzed	Range of Staphylococcal count/ml		Average Staphylococcal count/ml (mean value)	Standard deviation
		Maximum	Minimum		
Igloo	3	3.5	3.3	3.4	0.01
Polar	3	0	0	0.0	0.0
Kwality	3	0	0	0.0	0.0

All counts are expressed are logarithms

Table 6.
Statistical analysis of ice-cream brand samples in relation to Total viable, *E. coli* and Staphylococcal counts.

Types of ice cream	Total viable count	*E. coli* count	Staphylococcal count
Igloo	11850 b	9255 a	2595 a
Polar	13855 a	1136 b	0.0
Kwality	8530 c	794 c	0.0
Sd	2.2	3.9	1.2

* a to c rank order (highest - lowest)

Table 7.
Ratio relationship between total viable bacterial count and *E. coli* density per/ml portion of ice cream samples of different brands.

Brand	No. of sample analyzed	Average total viable count/ml	Average *E. coli* count/ml	Ratio of *E. coli* count : TVC
Igloo	3	11850	9255	1 : 1.2804
Polar	3	13855	1136	1 : 12.1963
Kwality	3	8530	794	1 : 10.7430

Table 8.
Ratio relationship between total viable bacterial count and staphylococcal density per/ml portion of ice cream samples of different brands.

Brand	No. of sample analyzed	Average total viable count/ml	Average staphylococcal count/ml	Ratio of *staphylococcal* count : TVC
Igloo	3	11850	2595	1 : 4.5664
Polar	3	13855	0.0	1 : 0
Kwality	3	8530	0.0	1 : 0

4.2 ISOLATION AND IDENTIFICATION

4.2.1 Bacterial count

4.2.1.1 Plate count agar for total viable count

The average counts/ml of Igloo, Polar,and Kwality were 1.19 x 10^4 CFU/ml , 1.39 x 10^4 CFU/ml and 8.53 x 10^3 CFU/ml respectively (Plate 3).

4.2.1.2 Eosin Methylene Blue (EMB) agar

The average *E. coli* counts/ml of Igloo, Polar and Kwality were 9.26 x 10^3 CFU/ml , 1.14 x 10^3 CFU/ml and 7.95 x 10^2 CFU/ml respectively (Plate 4).

4.2.1.3 Staphylococcus agar no.110

The average *staphylococcal* counts/ml of Igloo, Polar and Kwality were 2.60 x 10^3 CFU/ml, 0.0 CFU/ml and 0.0 CFU/ml respectively (Plate 5).

4.2.1.4 Identification of *Escherichia coli* by different bacteriological methods

4.2.1.4.1 Results of cultural examination

4.2.1.4.1.1 MacConkey (MC) agar

E. coli produced bright pink colored colonies on Mac Conkey agar (Plate 7).

4.2.1.4.1.2 Eosin methylene blue (EMB) agar

EMB agar plates streaked separately with the organism revealed the growth of bacteria after 24 hours of incubation at 37^{0}C aerobically. The growth was indicated by smooth, circular, black color colonies with metallic sheen on the agar plate (Plate 8).

4.2.1.4.1.3 Results of Gram's staining

The microscopic examination of Gram's stained smears from MC and EMB agar revealed Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain (Plate 9).

4.2.1.4.2 Results of biochemical test

4.2.1.4.2.1 Fermentation reaction with five basic sugars

The isolated bacteria fermented the five sugars (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) with the production acid and gas. The change of color from reddish to yellow indicated acid production. The presence of gas bubble in the inverted Durham's tube indicated gas production (Plate 10).

4.2.1.4.2.2 Triple sugar iron (TSI) agar slant test

In TSI agar slant, yellow slant, yellow butt, presence of gas bubbles and absence of black precipitate (plate 11).

4.2.1.4.2.3 Motility, Indole, Urease (MIU) test

Escherichia coli in MIU medium causing turbidity and urease production with indole positive (Plate 12).

4.2.1.4.2.4 Other biochemical tests

All the isolates were indole test positive, methyl-red positive and VP test negative. Results of these tests are shown in Table 9 and Plate 13, 14, and 15.

Table 9. Result of biochemical test for *E. coli*

Different biochemical test	Result
Fermentation reaction with sugar	
Glucose	+
Sucrose	+
Fructose	+
Mannitol	+
Fermentation reaction with dulcitol	
Indole	+
MR	+
VP	-

4.2.1.5 Identification of *Staphylococcus* spp. by different bacteriological methods

4.2.1.5.1 Results of cultural examination

4.2.1.5.1.1 Staphylococcus agar no.110

The organisms were observed as golden yellowish colonies on Staphylococcus agar no. 110 (plate 16).

4.2.1.5.1.2 Results of Gram's staining

The organisms were observed as Gram-positive cocci arranged in grape like clusters (Plate 17).

4.2.1.5.2 Results of biochemical test

4.2.1.5.2.1 Fermentation reaction with five basic sugars

The isolated bacteria were fermented the five sugars (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) with the production of acid. The change of color from reddish to yellow indicated acid production (Plate 18).

4.2.1.5.2.2 Triple sugar iron (TSI) agar slant test

In TSI agar slant, yellow slant, yellow butt, absence of gas bubbles and absence of black precipitate (Plate 19).

4.2.1.5.2.3 Other biochemical tests

All the isolates were methyl-red positive, Voges-Proskauer test negative, Motility, Indole, Urease (MIU) test and indole test negative. Results of these tests are shown in Table 10 and Plate 20, 21, 22 and 23.

Table 10. Result of biochemical test for *Staphylococcus*

Different biochemical test	Result
Fermentation reaction with sugar	
Glucose	+
Sucrose	+
Fructose	+
Mannitol	+
Fermentation reaction with dulcitol	
Indole	-
MR	+
VP	-

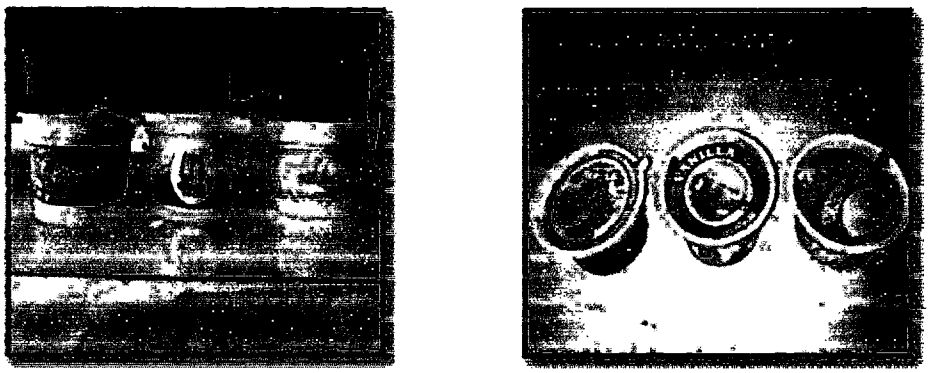
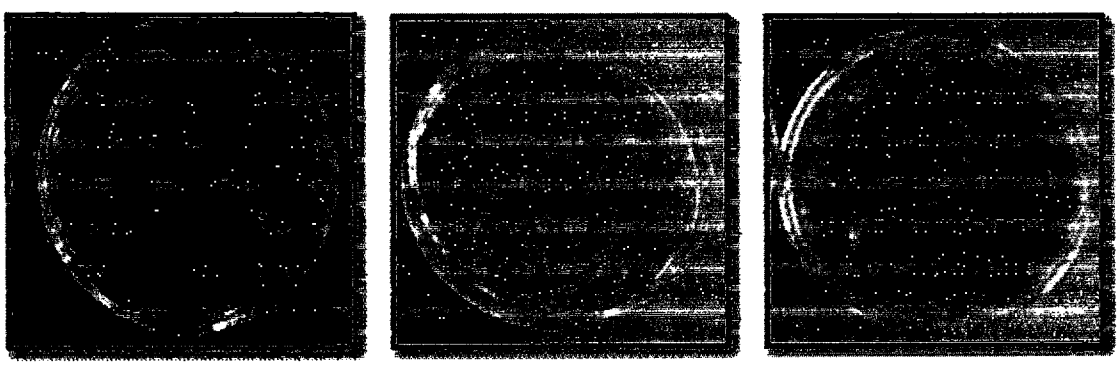


Plate 1: Sample of ice cream.

Plate 2: Ten fold dilution of ice cream sample.



(a) (b) (c)

Plate 3: Colony of bacteria in plate count agar for TVC (sample: a=igloo. b= polar. c= kwality).

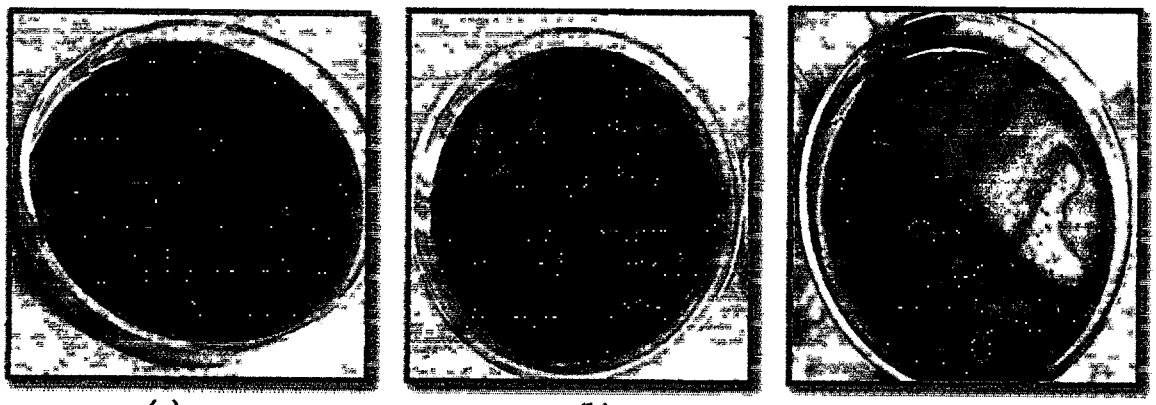


Plate 4: Colony of bacteria in EMB agar for total *E. coli* count(sample: a= igloo. b= polar. c= kwality).

Plate 5: Colony of bacteria in Staphylococcus agar no. 110. for total Staphylococcal count (sample: igloo).

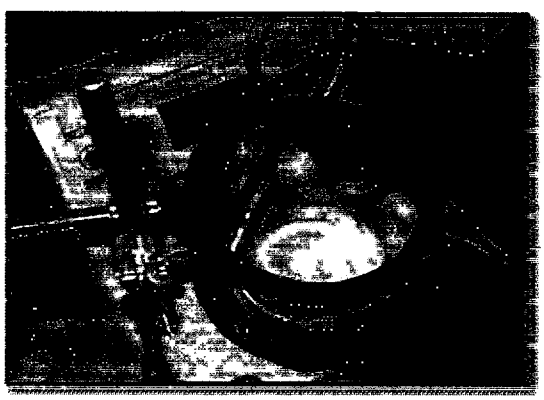


Plate 6: colony count in counter machine.

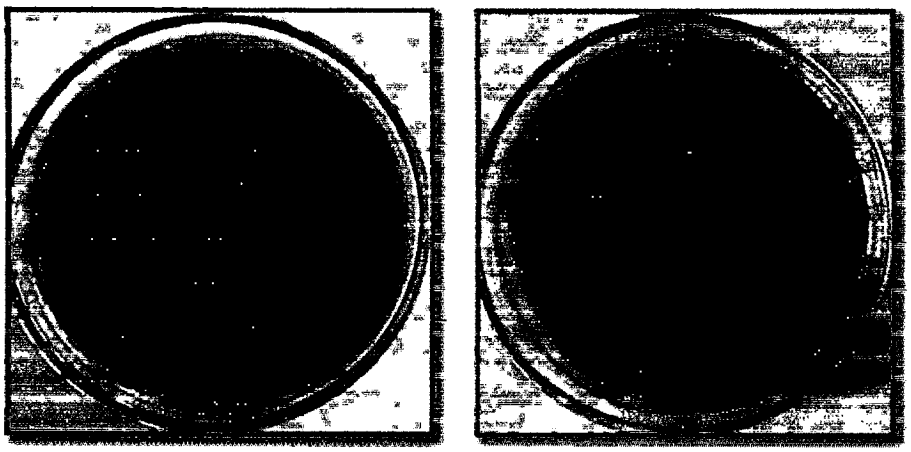


Plate 7: *E. coli* produced bright pink colored colonies on MacConkey agar (left) and uninoculated control (right).

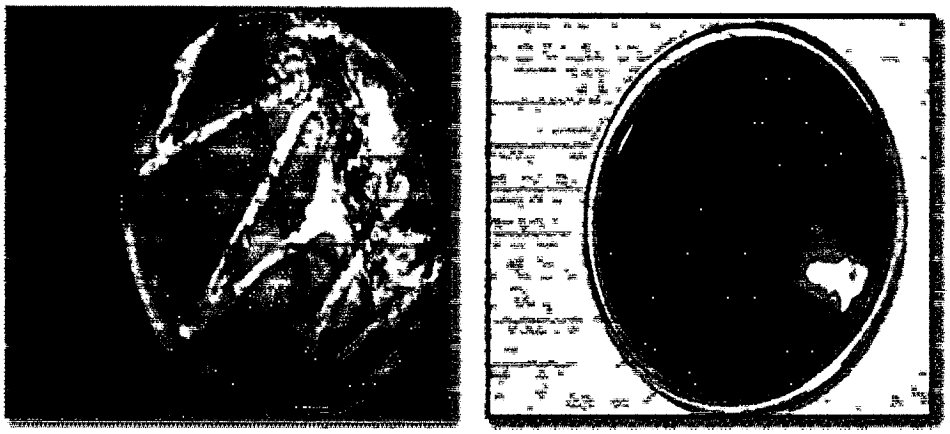


Plate 8 : Metallic sheen produced by *E. coli* on EMB agar (left) and uninoculated control (right).

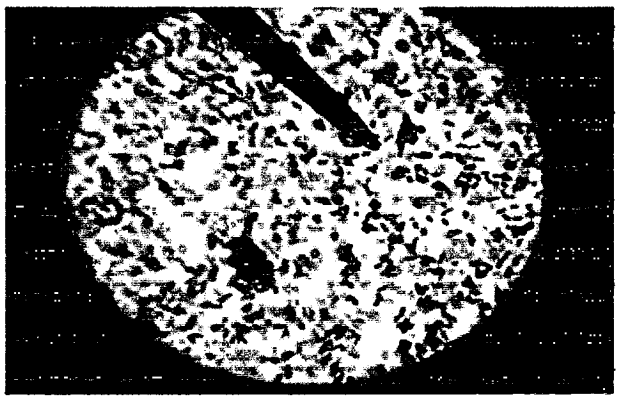


Plate 9: *E. coli* showing Gram negative (pink colour) small rod-shape, arranged in single or pair at 100x magnification (Gram's staining).

Plate 10: Fermentative activity of *E. coli* with five basic sugars (Dx, ML, L, S, MN AND C) with production of acid and gas (left) and uninoculated control (right).

Plate 11: Culture on triple sugar iron (TSI) agar slant reaction showing black color butt (right) indicate H_2S production by *E. coli* and uninoculated control (left).

Plate 12: Motility Indole Urease (MIU) test causing turbidity and urease production with indole positive by *E. coli* (right) and uninoculated control (left).

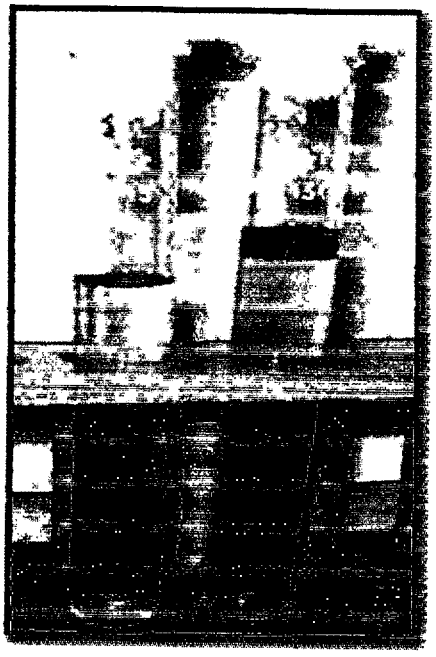


Plate 13: Indole test showing positive results with a red color in the reagent layer indicating indole production with reaction of *E. coli* (right) and uninoculated control (left).

Plate 14: Methyl-Red test for *E. coli* showing the medium was changed to bright red color (right) and uninoculated control (left).

Plate 15: Voges-Proskauer test for *E. coli* showing no change of the medium (left) and uninoculated control (right).

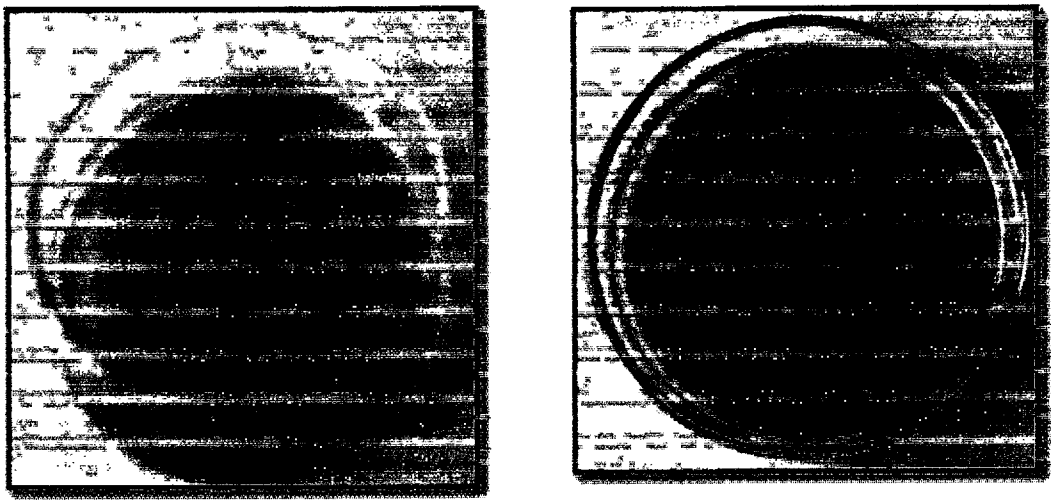


Plate 16: Culture of *Staphylococcus* spp. on Staphylococcus agar no. 110. (left) showing yellowish colonies and uninoculated control (right).

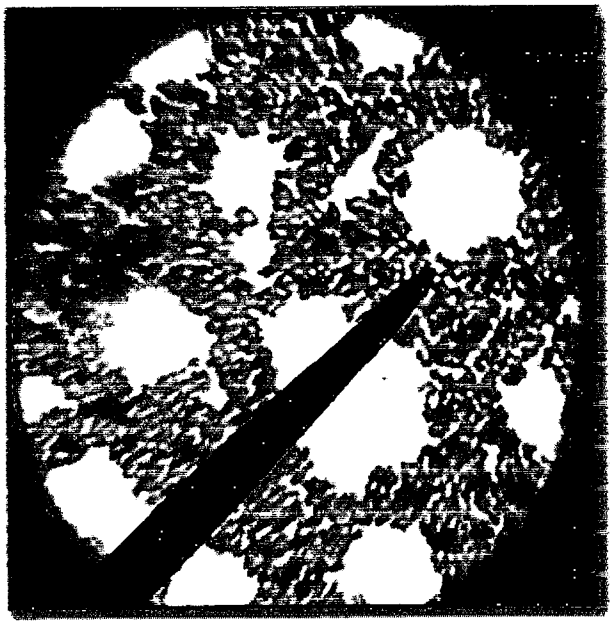


Plate 17: *Staphylococcus* spp. showing Gram positive (violet color) cocci arranged in groups or grape like- clusters at 100x magnification (Gram's staining).

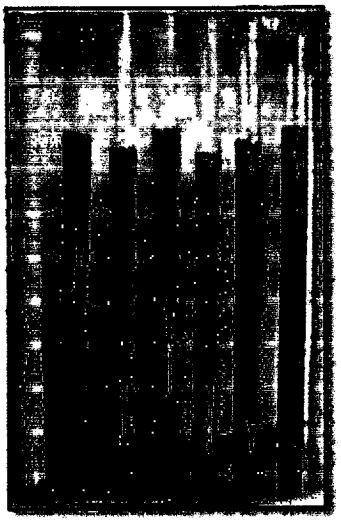


Plate 18: Fermentative activity of *Staphylococcus* spp. with five basic sugars(Dx, ML, L, S, MN and C) with production of acid (left) and uninoculated control (right).

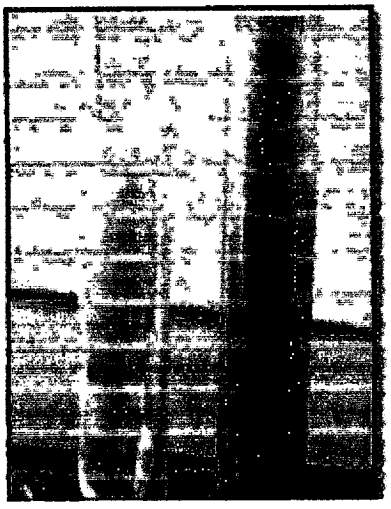


Plate 19: Culture on Triple Sugar Iron (TSI) agar slant reaction showing yellow slant and yellow butt (left) and production of no gas by *Staphylococcus* spp. and uninoculated control (right).

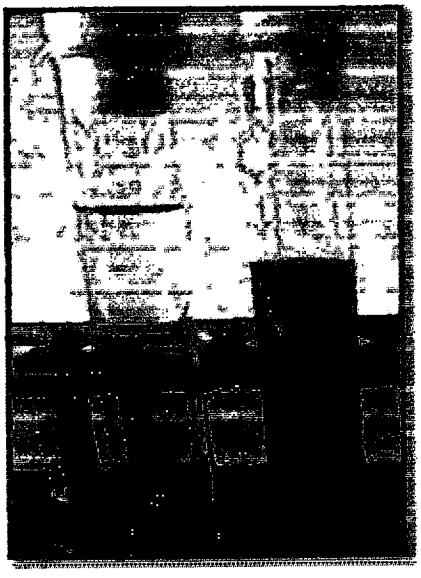


Plate 20: Methyl-Red test for *Staphylococcus* spp. showing the medium was changed to bright red color (right) and uninoculated control (left).

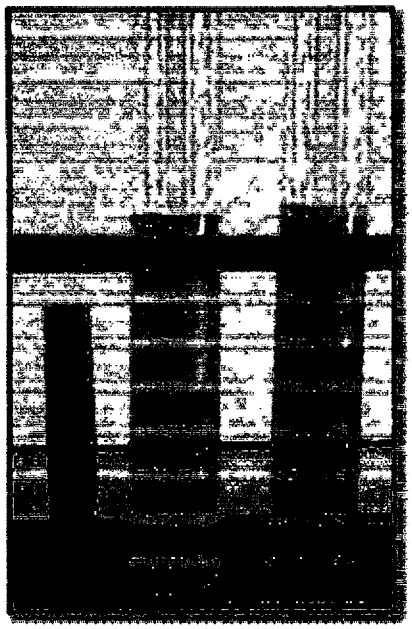


Plate 21: Voges-Proskauer test for *Staphylococcus* spp. showing no change of the medium (right) and uninoculated control (left).

Plate 22: Motility Indole Urease test causing no turbidity and urease production with indole negative by *Staphylococcus* spp. (right) and uninoculated control (left).

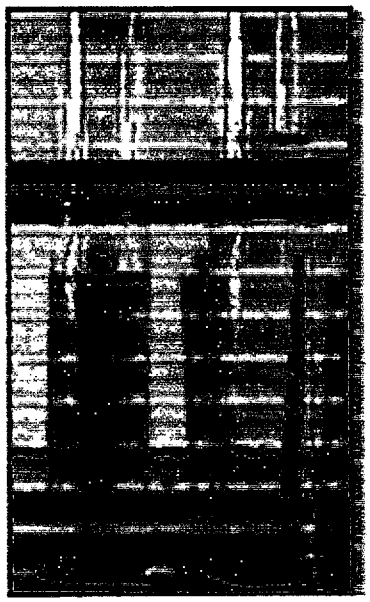
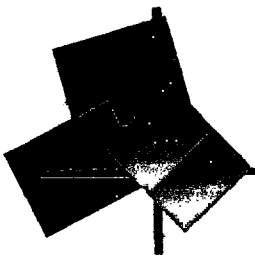


Plate 23: Indole test showing no change of the medium with the reaction of *Staphylococcus* spp. (left) and uninoculated control (right).



Chapter 6

discussion

CHAPTER 5

DISCUSSION

The present study showed that *E. coli* counts were the highest in igloo and the lowest in kwality ice cream (Table 2). The average count/ml were Igloo log 4.0, Polar log 3.0 and Kwality log 2.9, respectively (Table 4). It is evident from the result that the average *E.coli* count was 3.72 x 10^3 CFU/ml (log 3.3) (Table 2). The *E. coli* standards for ice cream should not be over 10/ml. The present investigation showed significantly high *E. coli* counts in samples belonging to Igloo, polar and Kwality ice cream. However, this study demonstrated that the samples of kwality and Igloo met the recommended criteria of USPHS. Hence, it could be taken into consideration as superior quality ice cream. It is known that *E. coli* play role in determining the hygienic quality index of food. In this study, *E. coli* counts positively and significantly correlated with total viable count of different brands of commercial ice cream. The average Staphylococcal counts/ml in different brand of ice cream samples were Igloo 2.60 x 10^3 CFU/ml (log 3.4), Polar 0.0 CFU/ml (log 0.0), and Kwality 0.0 (log 0.0) (Table 2). The maximum and minimum ranges varied from log 3.5 to log 3.3 in igloo , log 0.0 to log 0.0 in polar, log 0.0 to log 0.0 in kwality ice cream, respectively (Table 5). The average Staphylococcal counts of all three brands of ice cream was 8.65 x 10^2 CFU/ml (log 1.13) (Table 2). However, the highest count was observed in igloo ice cream sample and did not obtain in polar and kwality ice cream sample. In certain situation *Staphylococci* organisms particularly *S. aureus* may be a pathogen, a source of enterotoxin and indicator of unsanitary practice. In man, the main reservoir of *S. aureus* is the nasal cavity and skin. From these sources Staphylococci find their way into air and dust, into clothings and in other place from which foods get contaminated. Since Staphylococcal food poisoning is an intoxication and depends on the ability of food

concerned to support the growth of the Staphylococci which produce the toxin. It is therefore important to consider that the processing and handling of the food products should be so designed to minimize contamination and to make unfavourable medium for the growth of these organisms. When susceptible foods are produced with low numbers of Staphylococci, they will remain free of enterotoxin if kept either below until consumed.

The factors that contribute mostly to Staphylococcal food-borne outbreaks may be due to inadequate refrigeration, preparing food far in advance of planned service, infected persons practicing poor personal hygiene, inadequate heat processing and holding food in warming devices at bacterial growth temperature. Staphylococci may come into milk and milk product from food handlers who may have acute infections or from healthy carriers who harbor the organisms in their nose or throats and also it is due to improperly stored and refrigerated milk and milk product that make excellent culture media for growth of these organisms.

From Table 6 Considering the total viable count, *E. coli* count and Staphylococcal count the ice cream samples of Kwality ranked into 'c', Igloo 'b' and Polar sample 'a'. The analysis from the bacteriological context demonstrated that the lower count of bacteria in any food product signified comparatively better quality product, of course it should have to meet with international standard. In this respect ice cream sample of Kwality and Igloo were the best quality product and also it fulfilled the gradation 'c' and 'b'. Polar sample was of poor quality and graded into 'a' categories. In case of staphylococcal count, Polar and Kwality samples ranked into 'c' Igloo samples 'a'. It is also numerically arranged and in this context Kwality and Polar samples were of the best quality product. The results demonstrated that Kwality ice cream is the best quality product in respect of bacteriological context as well as hygienic point of view. In this study, a ratio relationship obtained between total viable count and *E. coli* count of three brands of ice cream, separately. These

were in case of Igloo 1: 1.2804, Polar, 1: 12.1963 and Kwality 1: 10.7430 (Table 7). From this result, a conclusion could be drawn that higher density of total viable count always contained less number of *E. coli*, since *E. coli* is the index of sanitary quality, the ice cream samples of Igloo were best and also Kwality have been found to possess the property of hygienically produced ice cream. In this study, Staphylococcal counts were positively and significantly correlated with total viable count in different brands of commercial ice cream, These were in case of Igloo 1: 4.5664, Polar, 1: α and Kwality 1: α (Table 8). Microbial quality of ice cream examined in Nepal by Joshi *et al.*(2004) in Hongkong by Food and Environmental Hygiene Department, in Phillipines by Orallo *et al. (1999)* and in India by Bhusan Reddy *et al.(1994)* also reported the more or less comparable results.

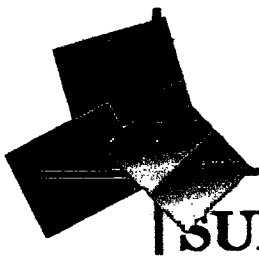
The frequency distribution of different species of bacterial isolates in different ice cream samples were found variable. Results of the present study indicate that all the two different types of bacteria were not present in the same ice cream sample collected from local market.

Characterization results of the study indicated that the ice cream samples contained Gram positive nonmotile and Gram negative motile organisms, colony characteristics in special media and fermentation ability with five basic sugars were similar. An interesting finding of the colony characteristics of the isolates were observed. The ice cream isolates were able to produce yellowish colonies on Staphylococcus agar no. 110, characteristic metallic sheen colony on EMB agar and bright pink colored colony on MacConkey agar.

In Gram's staining, the morphology of the isolated bacteria exhibited Gram positive (violet colour) cocci arranged in groups or grape like clusters; short cocco bacilli or rods arranged in bundles and singly also and Gram negative (pink colour) small

rod-shape, arranged in single or pair which were supported by several authors (Buxton and Fraser, 1977).

In the present study, biochemical tests which were used for characterization of bacterial pathogens also used by Ali *et al.* (2008). Among two isolates, *E. coli* produce acid and gas by fermenting various sugars and gave positive reaction to indole, Motility Indole Urease, Methyl red and Catalase test but negative reaction to Voges Proskeur test which satisfy the statement of Buxton and Fraser. (1977). *Staphylococcus* spp. produce acid but no gas by fermenting various sugars and gave positive reaction to Catalase and Methyl red test but negative reaction to indole and Voges Proskeur test.



Chapter 5

SUMMARY AND CONCLUSIONs

CHAPTER 6

SUMMARY AND CONCLUSIONS

The present study was conducted for the isolation, identification and determination of bacterial loads in ice cream collected from local market from Dinajpur during the period July to December, 2012. A total of nine samples of three brands-Igloo, Polar and Kwality were collected.

A series of tests were performed for the isolation, identification and total viable count of different types of bacteria and to determine the frequency distribution. Different types of ordinary, enriched and selective media such as nutrient agar, Plate count agar, MacConkey agar, eosin methylene blue agar, triple sugar iron agar, Nutrient agar slant and Triple Sugar Iron agar slant were used for the determination of the cultural characteristics of the different types of isolates. Biochemical properties of the isolated bacteria were studied by sugar fermentation, catalase, coagulase, MR, VP and indole tests.

From the present study, it was concluded that Igloo and Kwality ice cream are of the superior quality product in respect of sanitary condition in Bangladesh. However, much attention is still needed to apply aspects of microbiological quality control for attaining desired safety margins and giving assurance that the ice cream product received by the consumer will be pure, healthful and of the quality claimed. To do so useful and effective sound legislation must have to be enacted and enforced, the chief aim of which is to ensure that the production, handling, processing, distribution and storage of ice cream could be maintained under strict hygienic control to protect consumers against health hazard and under quality standards.

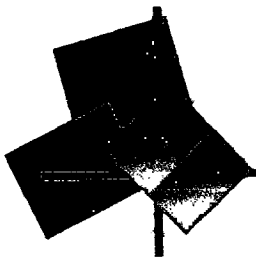
In the context of this study, it may be concluded that,

i. Total viable count of organisms was successfully performed from different ice cream samples.

ii. *E. coli* and *Staphylococcus* spp. were present in most of ice cream and could be isolated from ice cream samples.

iii. *E. coli and Staphylococcus* was successfully isolated and confirmed by different bacteriological agar media and biochemical reaction.

iv. Hygienic measure should be taken to ensure that the production, handling, processing, distribution and storage of ice cream.



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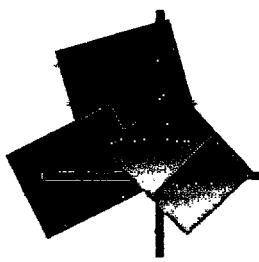

APPENDICES

4. TSI agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	10.00
Casein enzymic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00
Final pH(at 25°C)	7.4 ± 0.2

5. MIU medium base (Hi Media)

Ingredients:	**g/L**
Casein enzymic hydrolysate	10.00
Dextrose	1.00
Sodium chloride	5.00
Phenol Red	0.01
Agar	2.00
Final pH(at 25°C)	6.8 ± 0.2

6. MR-VP medium (Hi Media)

Ingredients:	**g/L**
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH (at 25°C)	6.9 ± 0.2

7. Sugar media

Ingredients:	**g/L**
a. Peptone water	
Bacto-peptone	10.0 gm
Sodium chloride	5.00 gm

0.5% phenol red	0. 10 ml
Distilled water	1000 ml

b. Sugar solutions

Individul sugar	5.00 gm
Distilled water	100 ml

c. Sugar media preparation

Pepton water	4.50 ml
Sugar solution	0.50 ml

8. Peptone water

Ingredients:	**g/L**
Peptone	1.00 gm
Distilled water	1000 ml

APPENDIX 2

Preparation of reagents

1. Kovacs reagent

P-dimethyl aminobenzal dehyde	5 gm
Amylalcoho	175 gm
Conc.HCL	25 ml

2. V-P reagent 1

5% alpha –naptholin absolute ethyl alcohol

3. V-P reagent 2

40%potassium hydroxide containing 0.3 creatine. The ingredients were dissolved by heating gently over steam bath. When in solution add 0.05gm of cotton blue dye.

4. Phosphate buffered solution

Sodium chloride	8 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water to make	1000 ml

5. Methyl red solution

Methyl red	0.05 gm
Ethanol (absolute)	28 ml
Distilled water	22 ml

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Potassium hydroxide solution

40% aqueous solution of KOH

8. Gram stain solution

□ **Stock crystal violet**

Crystal violet	10 gm
Ethyl alcohol (95%)	1000 ml

□ **Stock oxalate solution**

Ammonium oxalate	1 gm
Distilled water	1000 ml

□ **Lugols iodine solution**

Iodine crystal	1 gm
potassium iodide	2 gm

□ **Ethyl alcohol** 250 ml

□ **Acetone** 250 ml

□ **Counterstain**

Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranine working solution
The stock safranine is diluted 1:4 with distilled water.