Isolation of Bacteria from Dairy-based Popular Sweetmeat named Swandesh following the Molecular Identification of *Bacillus* Species

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFIL MENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by
Nazoa Shimin Tui
Student ID: 13136014

Biotechnology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Declaration

I hereby declare that the thesis project titled "Isolation of Bacteria from Dairy-based Popular Sweetmeat named Swandesh following the Molecular Identification of *Bacillus* Species" has been written and submitted by me, Nazoa Shimin Tui and has been carried out under the supervision of Kashmery Khan, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

(Nazoa Shimin Tui)

Candidate

Certified by

(Kashmery Khan)

Supervisor

Lecturer

Biotechnology Program Department of Mathematics and Natural Sciences

BRAC University, Dhaka

Dedicated

to

My Parents

Acknowledgement

First and foremost, I would like to express my thanks to Almighty Allah because He has given me the opportunity and strength to finish this research. I am also thankful for His blessings to my daily life, good health and healthy mind.

I acknowledge my esteem to Professor **A F M Yusuf Haider**, Chairperson of MNS Department of BRAC University for giving me the opportunity to complete my undergraduate thesis. I am really grateful to Professor **A. A. Ziauddin Ahmad**, former Chairperson of MNS department of BRAC University.Professor **Shah M. Faruque**, **Dr. Aparna Islam, Dr. Mahboob Hossain** of MNS Department of BRAC University for allowing me and encouraging me to complete my undergraduate thesis.

My regards, gratitude , respect and indebtedness goes to my respected supervisor **Kashmery khan**, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC Universityfor her guidance, suggestions ,constructive criticism , enthusiastic encouragement , providing new ideas throughout my thesis work.

I would like to thank and express my deepest gratitude toward **Nahreen Mirza** for guiding and helping me in my report writing and providing time to time suggestions regarding setting of experimental designs, interpretation of results and subsequent directions for the whole work without being a bit of impatient.

I would also like to thank **Salman Khan Promon** and **Asma Binte Afzal** for their time and support throughout my thesis work.

I also express my special thanks to all my respected teachers at the Department of Mathematics and Natural Sciences, BRAC University, for their academic counsel and encouragement.

I would also like to specially thank my friend Mourin and Kanon for their support and help in my work.

Finally, I would like to extend my gratitude to my parents, Dr. Mahabubul Islam Majumder and Shahana Parveen without whose guidance this endeavor would have been impossible. I would also thank the members of my family and to my friends for their prayerful concerns and supports.

Nazoa Shimin Tui

May, 2018

Abstract

Among all the sweetmeats found in Bangladesh, Swandesh achieves an intense popularity due to its delicate texture and sublime taste. Like other dairy products, it is high in carbohydrates and proteins, along with probiotic contents. However, the presence of pathogenic bacteria in Swandesh put them at a grave risk for public health. Therefore, it is highly crucial to evaluate the microbial contamination in it. Current study was done to check the status of several microorganisms in Swandesh. After isolation and identification, the most prevalent microorganism in Swandesh was found out as *Staphylococcus* species followed by *Bacillus* species. The samples were then demographically represented which showed Mohakhali and Dhanmondi having the highest number of microbial isolates. Later, the antibiotic sensitivity testing was done to check the effectivity of antibiotics against the isolates. Where ciprofloxacin was effective against 100% of the isolates. On the other hand, all the isolates were resistant of cefepime. The isolates of *Bacillus* which showed the highest amount of resistance, were taken for PCR and both samples gave bands at about 1500 bp, which corresponds to their band size for 16s rRNA amplification. This shows the effectivity of the PCR method for rapidly detecting *Bacillus* from food samples.

Table of Contents

Chapter: 1

Introduction

Introduction

1.1 Background

The number of foodborne diseases is increasing alarmingly. Foodborne diseases are responsible for many people's morbidity and even mortality. Even though our quality of life is increasing to most extents, when it comes to food processing, the quality often lags. This is more seen in the developing and underdeveloped countries. Due to lack of regulation and awareness about general hygiene, food products are frequently contaminated with microbes. Some of these microbes can cause disease and eventually can even cause death.

The food products where potentially harmful bacteria inhabit are high in nutrition. Dairy products are very high in carbohydrates and protein, along with other vital nutrients. Whereas, this makes these perfect for growth and development, it can cause problems if not handled properly. The high level of nutrition provides an ambient environment for many pathological bacteria (Soomro *et al.*, 2003). Some bacteria help to bring new texture, taste and appearance to milk products like yogurt, butter and curd. These bacteria are lactic acid bacteria or other firmicutes. Pathogenic bacteria like *Escherichia coli, Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter jejuni*, *Salmonella* species, *Listeria monocytogenes* and some more (Amani *et al.*, 2015).These are found in both raw milk and processed milk products. *Staphylococcus aureus* poses great risk as it can produce heat tolerant toxin that will persist even after cooking (Prescott, 2002). Being a spore former organism, *Bacillus* spp also shows great likely hood of remaining in cooked dairy products. Faecal coliforms like *E. coli* are also found in dairy products as a result of using contaminated water in the preparation of these food (Sen & Rajoshia, 1989).

The cooked dairy products that are available in the country are Roshogulla, Swandesh,Yogurt, Kalojaam, Rashmalai etc. Apart from being high in food value, these sweets are also delicious in terms of taste. Children are a major consumer of these products and plain milk. They are often susceptible to many diseases of the gastrointestinal or respiratory tract. The presence of pathogenic bacteria in dairy items put them at a grave risk. Therefore, it is highly crucial to evaluate the microbial condition of the dairy products and determine the predominant organisms.

1.2 Swandesh: Composition and Tendency to Spoilage

Swandesh is a highly popular sweetmeat of our country. Its delicate texture and sublime taste has made it a favourite to everyone. Sadly, the high nutrition and low sugar content has made it a favourite of bacteria as well. It is very easy for some bacteria to grow in them (Tamang*et al*., 2016). The process of Swandesh making involves production of cottage cheese and then cooking it with sugar. Microbial contamination can take place at any of its processing stages. The microbes can grow on the raw milk used to prepare the cottage cheese (Ogier, 2002). However, in cottage cheese, there is a high number of probiotic bacteria like *Lactobacillus* that often prevents it from spoiling (Khan & and Kang, 2016). During this step bacteria can encounter it. Additionally, while moulding the *Swandesh* pieces, hands of the maker is used. If

their hands are not clean, then many bacteria residing on skin can pass on to the Swandesh. Moreover, improper packaging and processing can also allow bacteria to grow (Pal *et al*., 2016).

1.3 Microorganisms commonly found in dairy products

As previously mentioned, due to the high sugar and protein content, microbes can easily inhabit dairy products. These microbes are often heat tolerant, spore forming or at least produce toxins that are heat stable. The most common microorganisms found in dairy products (Oliver *et al*., 2005) are discussed below.

1.3.1 *Bacillus* species

Even though mostly harmless, *Bacillus* species can cause several diseases when consumed. *Bacillus cereus* can cause both diarrhoea and vomiting. It is an aerobic microorganism that can grow very well under anaerobic conditions. Due to spore forming capabilities, it can remain in food even after it has been cooked or refrigerated (Larsen, *et al*., 1996). It can very well grow in the intestines where it produces enterotoxins that can cause diarrhoea. While the *B. cereus* in food causes emitting toxins. The heat treatment given to kill pathogenic microbes allow the dormant spores of *Bacillus* to germinate. Moreover, there has been emergence of some psychrotropic strains of *B. cereus* that can survive in cold temperature. Therefore, even if the dairy products are refrigerated, these microbes can sustain (Logan, 2011)

The taxonomic hierarchy of *Bacillus* given below.

Kingdom : Bacteria

Phylum : Firmicutes

Class : Bacilli

Order : Bacillales

Family : Bacillaceae

Genus : *Bacillus*

1.3.2 *Staphylococcus aureus*

Another microbe that is predominant in food borne diseases is *Staphylococcus aureus*. It is gram positive, salt tolerant and commonly found on skin. However, some of its strains are pathogenic as they can produce harmful toxins. These toxins can cause gastroenteritis that is an inflammation of the intestines resulting in pain, vomiting and diarrhoea (Payne & Wood, 1974). Even though, *S. aureus* is heat killed, its toxins are highly heat tolerant. Apart from the gastrointestinal diseases, *S. aureus* can also cause infection of the skin, toxic shock syndrome, endocarditis, septicaemia and so on (Soomro*et al*., 2003). As it is found on skin, it can come in contact with the dairy food while processing (Cogen *et al*., 2008)

The taxonomic hierarchy of *Staphylococcus* given below.

Kingdom : Bacteria

Phylum : Firmicutes

Class : Bacilli

Order : Bacillales

Family : Bacillaceae

Genus : *Staphylococcus*

1.3.3*Escherichia coli*

Escherichia coli is one of the most common microorganisms in this world. They are often associated with fatal diseases resulted from diarrhoea (Pawlowski*et al.,* 2009). *E coli* are gram negative, non-spore forming, facultative anaerobe with rod shape (Tortora, 2010). By producing many endotoxins, *E coli* can infect human and cause gastrointestinal diseases (Checkly*et al*., 2008). These can also cause urinary tract diseases along with septicaemia (Toder, 2007). The pathogenic *E.coli* include Enterotoxigenic *E.coli.* (ETEC), Enteropathogenic *E coli.* (EPEC), Enteroinvasive *E coli.* (EIAC), Enterohemmoregic *E coli.* (EHEC), Enteroaggregative *E coli.* (EAEC) and Adherent Invasive *E coli.* (AIEC) (Evans *et al*., 2007).

Kingdom :Bacteria

Phylum :Proteobacteria

Class :Gammaproteobacteria

Order: :Enterobacteriales

Family :Enterobacteriaceae

Genus :*Escherichia*

Species :*E. coli*

1.3.4 *Salmonella* species

Another common enteric pathogen is *Salmonella* species. It is a gram negative rod shaped bacteria found in the enterics. It has only two species, which are divided into many sub species and numerous serotypes (Tortora, 2010). It can cause typhoid and paratyphoid, which can be often lethal (Su & Chiu, 2008). *Salmonella* is often found in food and cause massive damage to the person consuming it (Swaminathan*et al*., 1978).

Domain	:Bacteria
Kingdom	:Eubacteria
Phylum	:Proteobacteria
Class	:Gammaproteobacteria
Order	:Enterobacteriales
Family	:Enterobacteriaceae
Genus	:Salmonella

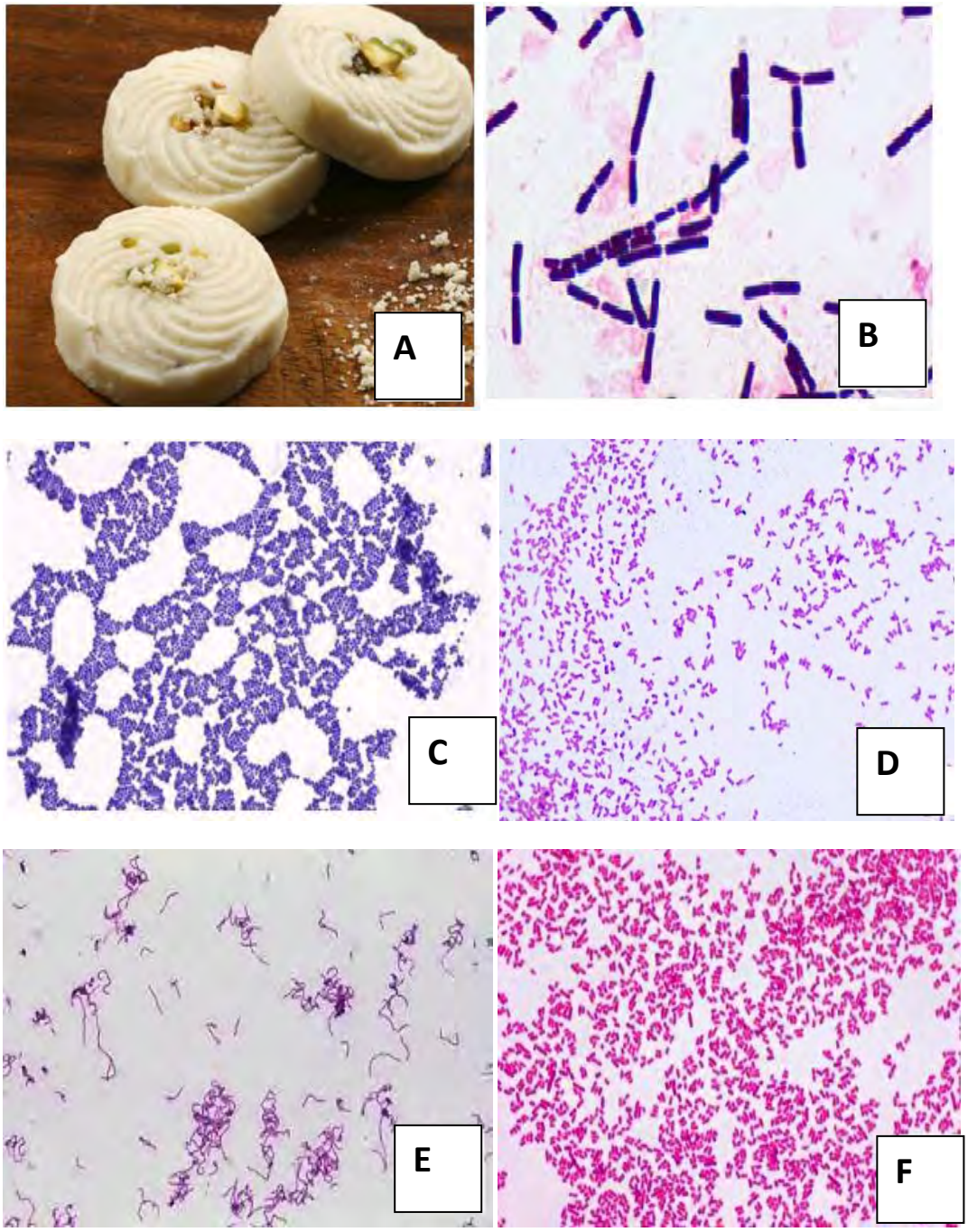

Figure A: Swandesh sold in shops of Bangladesh; Figure B: Microscopic image of *Bacillus* spp

Figure C: Figure 3: Clustured structure of *Staphylococcus aureus* seen under microscope;

Figure D: Microscopic image of *E.coli; Figure*E:Microscopic image of *Lactobacillus* spp ;

Figure F: Microscopic image of *Salmonella*spp

1.3.5 *Lactobacillus* species

Lactobacilli are a group of gram positive rod shaped bacteria that can produce lactic acid. These microbes have natural probiotic activity. *Lactobacilli* can adhere to the cell linings, reduce nutrition available to pathogenic microbes and produce bacteriocin. As a result, they are considered beneficial microbes (Reid *et al.,* 1999). In dairy products, they are found abundantly and prevent spoilage (Salminen*et al*., 1996).

Domain :Bacteria

Phylum :Firmicutes

Class :Bacilli

Order :Lactobacillales

Family :Lactobacillaceae

Genus :Lactobacillus

1.4 Antibiotic resistance

Antibiotics are a group of life saving drugs that can treat bacterial infections. The above-mentioned organisms can cause infections in human that can be treated by antibiotics. These organisms are gram positive and the antibiotic groups that has been used to treat Gram positive infections are as follows: first generation cephalosporins, carbapenems, glycopeptides, lincosamides, lipopeptides, quinolones and some more (Green, 2002). However, the bacteria are gradually acquiring resistance against the antibiotics used against those. They can secret enzymes to degrade the antibiotics or change the structure of the active site where antibiotics would have worked (Nikaido, 2010). It is very important to find out microbes that are resistant to different antibiotics. Moreover, it is crucial to use the most suitable antibiotic to treat any infection for proper treatment. Therefore, antibiotic sensitivity testing must be done (Reller*et al*., 2009).

1.5 Molecular level identification of species

Organisms can be identified by molecular level with many available techniques. In the bacterial genome, the 16s rRNA is distinctive of the species, due to slow rate of evolution in that region. After DNA extraction, carrying a polymerase chain reaction with the prime of 16s rRNA region, the identity of the organism can be found.

Polymerase chain reaction is an amplification process of DNA that is done *in vitro.* It was invented by Kary Mullis in 1983 (Mullis *et al*, 1986). Since then, after many modifications and improvements have taken place to allow rapid detection of nucleotide sequences. Then, after PCR is completed, the amplified DNA is passed through gel electrophoresis where, different sized DNA fragments can be separated in terms of their sizes.

1.6 Objective

The key to preventing foodborne diseases is to find out the microbial quality of the food samples. This will give an idea about the level of contamination in food items. Dairy items are compulsory food items for growing children and others. However, contaminated dairy items like Swandesh can create more problems if ingested. Therefore, it is vital to find out if any microbes are dwelling in these sweetmeats and what microbes are those. Additionally, finding out the antibiotic sensitivity patterns of the isolated organisms can be done..

This brings to the objective of the study

1. Isolation of microbes from Swandesh collected from various places of Dhaka city.
2. Identification of the isolated organisms.
3. Antibiotic sensitivity test of the isolated organisms for selected antibiotics.
4. Molecular identification of the most resistant isolates.

Chapter: 2
Materials & Methods

2. Materials and Methods

This study was held in the biotechnology and microbiology of BRAC University by following all the required instructions and by abiding all the rules of the institution.

2.1 Sample Collection

For this study *Swandesh* from different shops were collected. These shops were different in location. One piece of the Swandesh from each shop were collected in previously sterile packets.

Table 1*: Source of Swandesh from different shops of Dhaka*

	Area				
Shop-name	Mohakhali	Uttara	Dhanmondi	Greenroad	Jahangir Gate
	Bikrampur	Bismillah	Modhubon	Ovijat	Captain's
	Prime Sweets	The Alibaba	Muslim		
	Jolkhabar	Noni	Banoful		
	Rajvog				
	Bhaggokul				

Every sample was serially diluted within 24 hours of collection and then taken for isolation of organisms.

2.2 Serial Dilution

From each piece of Swandesh, 1gm was carefully measured maintaining sterility. Then it was added to physiological solution and mixed properly by using a vortex.

The physiological saline was prepared by mixing 0.85gm of sodium chloride with 100ml of distilled water. For every sample, 5 test tubes containing 9ml saline solutions were prepared and later autoclaved. These were labelled as: 10^{-1}, 10^{-2},10^{-3},10^{-4},10^{-5}. The Swandesh sample was added to 10^{-1} tube. Later, 1ml from each tube was transferred to the next tube after mixing thoroughly in vortex. This gave a 10-fold dilution. While transferring, micropipette was used, and the tips were changed every time the suspension was taken to another tube.

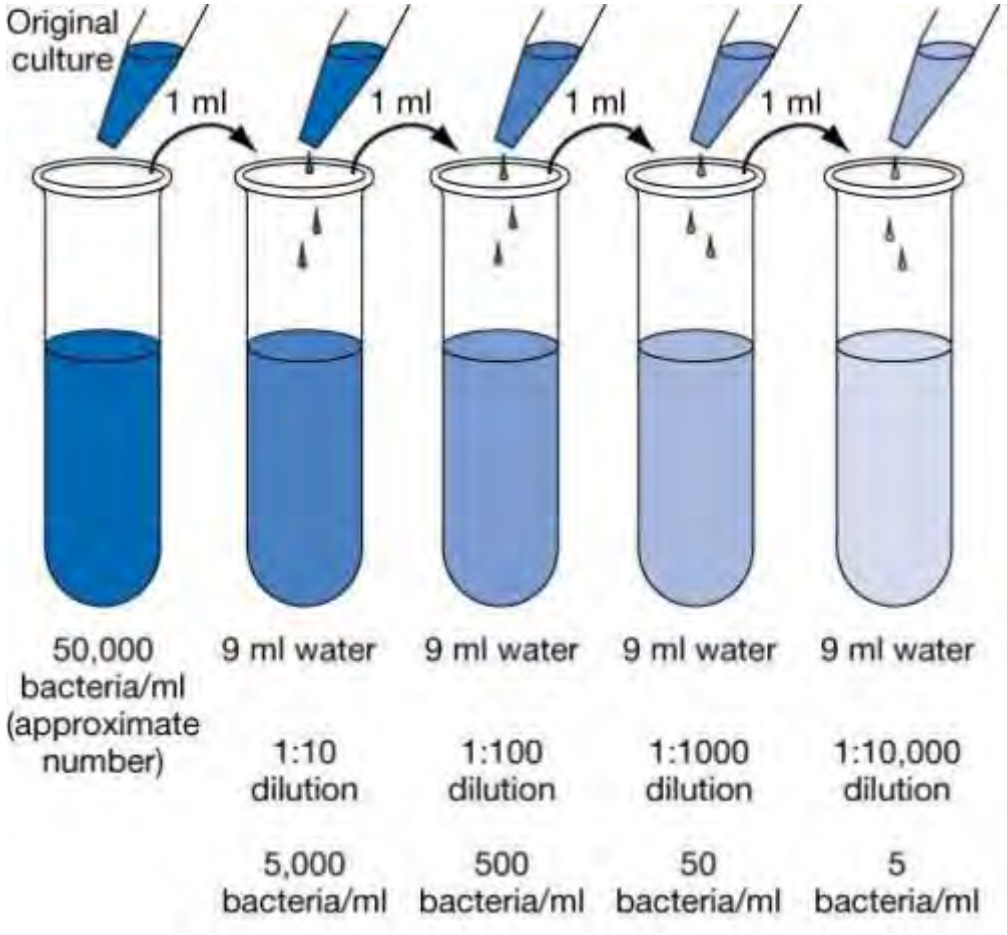

Figure 1*: Serial dilution of a given sample*

2.3 Nutrient Agar (NA) preparation

Nutrient agar is a basal growth medium for most non-fastidious organisms. In this study, it was used to isolate organisms from the saline suspension. For the preparation of NA, laboratory grade NA powder sold in correct composition was prepared. About 28gm of NA powder was added to 1000ml distilled water. It was then dissolved in a conical flask and boiled on burner until bubbles appeared. Later, the flasks were covered in small pieces of aluminium foil and kept in autoclave for sterilization.

After being autoclaved, the liquid medium was put in sterile dry petri dishes. For a large plate, 30ml medium was used. The plating was done inside the laminar airflow. Each plate was kept until the medium solidified. Later, the lids on the plates were put on and the plates were stored for upcoming use.

2.4 Microorganism Isolation

As previously mentioned, the Swandesh samples were mixed into fine saline suspension of different concentration. From each dilution, 0.15ml suspension was taken by a micropipette and placed on the nutrient agar plate. There were 5 plates for every sample. A glass rod was burned to red hot and then cooled off was used to spread the suspension evenly on the plate. Later, the plates were kept in the incubator set at 37^{o} for 24 hours. After incubation, the plates

were checked for any microbial growth. The plates containing colony numbers from 30-300 were used for isolation of the organisms. Distinct individual colonies were then streaked on NA plates for further work.

2.5 Identification of the organisms

The isolated colonies were streaked on selective media. For streaking, a loopful of organism was taken and placed on the media in such a way that a matte line was produced. Later, the loop was burned to red hot and cooled. From the matte line, 3-4 thinner lines were drawn on the direction of 90ºangle. The loop was again burned and cooled off. From the new streaked lines, 3-4 more lines were streaked at the same angle. This process reduced number of bacteria every time the loop was burned. Finally, the concentration was low enough to give individual separated colonies of the organisms that could be considered pure culture. The selective media used in the study is given below.

2.5.1. MRS Agar

The MRS agar media is used for identification and isolation of *Lactobacillus* spp. It was developed by de Man, Rogosa and Sharpe. Lactobacilli are non-spore forming, facultative anaerobic and gram-positive rods. They can be found in dairy products, foods and beverages. The MRS agar media show selective results for the Lactobacillus and provide proper nutrients. The media was prepared by adding 67.15 grams of MRS powder in 1000 ml of distilled water. After it was dissolved, it was then covered to be autoclaved. Later, it was plated into petri dishes and cooled to be hardened. The organisms from different sources were streaked afterwards.

2.5.2 Mannitol Salt Agar

This is another selective and differential medium. It is used to isolate Gram positive organisms that grow well in high salt concentration. This medium has a salt concentration of 7.5% that inhibits Gram negative organisms. It also has mannitol that some organisms can utilize, thus giving a different colour. MSA is used to select and differentiate *Staphylococcus* spp, *Streptococcus* spp. It is prepared by adding 111.02gm of powder in 1000ml of distilled water. With it is dissolved and then covered to be autoclaved. Later, it is plated into petri dishes and cooled to be hardened. The organisms from different sources were streaked afterwards.

2.5.3 Xylose Lysine Dextrose Agar

It is selective for Gram negative and differentiable for *Salmonella* spp. This is prepared by adding 56.68gm of the powder to 1000ml distilled water. Later by boiling with heat, it is dissolved. After dissolving, it is plated on petri dishes, hardened and streaked on.

2.5.4 Bacillus cereus Agar

This is selective for spore forming organisms. It is differentiable for *Bacillus cereus* or *Bacillus thuringesis*. The preparation includes addition of 20.5gm of powder base to 475ml distilled water. After boiling and keeping in autoclave for sterilization, it is slightly cooled and 25ml egg yolk emulsion is added along with one vial polymyxin B Supplement. Later, it is plated into sterile petri dishes and hardened to be streaked on.

2.5.5 Eosin Methylene Blue Agar

This medium is used to select and differentiate Gram-negative bacteria. It was prepared by adding 35.96gm of dry powder into 1000ml distilled water. It was put on burner and stirred regularly to dissolve the powder completely. Later, the flask containing the medium was covered with aluminium foil and taken to autoclave. After bringing the medium out of the autoclave, it was put on sterile petri dishes to be plated and hardened. Later, organisms from different sources were streaked by following the previously mentioned streaking method.

2.6 Biochemical tests

Biochemical tests are done identify an organism based on their biochemical preferences and pathway. The isolates that were found from selective media were passed through several biochemical tests to be confirmed about their identity. The following biochemical tests were done.

2.6.1 Methyl Red

Overnight culture of the organisms on nutrient agar were inoculated in sterile peptone water. A loop was burned to pick out a colony from the culture plate and then placed in peptone water. The suspension was kept in an incubator for another overnight growth. After bringing the peptone water out of the incubator with the bacteria, 2-3 drops of methyl red were added, and it was kept still for 5 minutes. Any occurrence of dark red colour indicated positive result, whereas, orange of yellow colour indicated a negative result.

2.6.2 VogesProskeur

Similar to the methyl red test, fresh cultures of the isolates were inoculated into peptone water. After overnight incubation, it was brought out and 2-3 drops of Barritte's A reagent were added. Then, 2-3 drops of Barritte's B reagent were added and kept for 15 minutes. A pink colour indicated positive result, no colour change meant negative result.

2.6.3 Citrate

Simmon's citrate agar was boiled in distilled water and then poured into small vials. After being autoclaved, it was kept at an angled position to create a slant. After the slants hardened, overnight culture of the isolates was streaked on the slant. It was kept at an incubator for overnight. On the next day, the slants were observed for colour change. Blue colour indicated positive result; green colour indicated negative result.

2.6.4 Indole

Peptone water was inoculated with a loopful of overnight culture of the organism. The suspension was kept in the incubator. On the next day, 2-3 drops of Kovac's reagent was added to each test tube. It was kept for 2 minutes to observe any colour change. Red colour meant positive result; yellow colour meant negative result.

2.6.5 Catalase

Catalase test is done by making 3% hydrogen peroxide solution and then placing it on a glass slide where a loopful of fresh culture was added. Bubble formation indicated positive result.

2.6.6 Motility Indole Urease

The base of Motility Indole Urease powder was added to distilled water and boiled on burner. While the medium remained liquid, it was poured in test tubes, and put in autoclave. After bringing out of the autoclave, sterile 40% urea solution was added to each tube at a 10% volume of the total volume. After the medium was hardened, fresh culture of the organism was stabbed on the agar with the help of a needle.

2.6.7 Triple Sugar Iron

The base powder of the Triple sugar iron was added to distilled water and boiled. Later, it was put in test tubes. Afterwards, it was autoclaved. While it remained warm, it was put on a slant position and hardened. With an inoculating needle, fresh culture was first stabbed and then streaked on the slant. It was observed for sugar fermentation and gas formation.

2.6.8 Gram Staining

Gram staining is the process of classifying bacteria in terms of their cell wall composition. From a fresh culture of the organism, loopful of bacteria is smeared onto a sterile glass slide. Later it is heat fixed and allowed to dry. A few drops of crystal violet were placed on the smear and washed off after 1 minute. Then, Gram's iodine was put on the smear for another minute. After washing the Gram's iodine, the smear was again washed with 95% ethanol to remove residual dye. Later, drops of safranin was placed on the smear for 30 to 45 seconds. After washing this last dye off, the slide was allowed to be dried. Lastly it was observed under the microscope for viewing the cell.

2.7 Antibiotic Sensitivity Test

Antibiotic sensitivity test was done on Mueller Hinton agar. It was prepared by adding 38gm of MHA powder in 1000ml distilled water. After boiling and sterilizing the medium, it was plated into petri dishes and allowed to set.

A loopful of bacterial culture was taken on a cooled sterile loop. It was added to a tube of physiological saline and mixed form an even suspension. Sterile cotton swab was then dipped into individual test tubes. After rinsing off the residual water, the swab was spread onto the entire plate as a lawn. Then, with sterile forceps, antibiotic discs were taken out and placed on the bacterial lawn. The plate was kept in the incubator for overnight. On the next day, the zone diameter for each antibiotic was measured.

2.7.1 List of antibiotics used

A total of 11 antibiotics from different groups were selected for antibiotic sensitivity testing. The list of antibiotics used in the study are given below

Table 2: List of antibiotics used for antibiotic sensitivity testing

Antibiotic	
1. Amoxycilin	7. Cefuroxime
2. Trimethoprim	8. Gentamicin
3. Norfloxacin	9. Cefixime
4. Cotrim	10. Vancomycin
5. Colistin	11. Azithromycin
6. Ceftriaxone	

2.8 Storing of the isolates in T1N1

The isolates were stored in T1N1 vials. The T1N1 was prepared by adding 1gm of tryptone casein digest, 1gm of NaCl and 0.6gm of agar powder. The mixture was boiled and poured into glass vials. Later, the vials were autoclaved and allowed to solidify. After it solidified, bacterial inoculum was taken on a sterile needle and stabbed on the media. It was kept in the incubator for overnight. On the next day, 300ul of sterile paraffin oil was added on top of the agar. The vials were tightly capped and stored in room temperature.

2.9 DNA extraction

The samples were grown overnight in nutrient broth and then were used to isolate the DNA. The wizard® Genomic DNA Purification Kit was used in to obtain genomic DNA from the potential experimental bacterial isolate. From the overnight culture, 1 ml from the broth was added to 1.5 ml micro centrifuge tube. Then it was centrifuged (Centrifuge; Eppendorf, Germany) at 13,500 RPM for 2 minutes to separate the cells. The bacterial pellet was resuspended, and DNA was extracted according to the protocol provided with the wizard® Genomic DNA Purification Kit. The DNA was stored at -20◦C until use.

2.10 Polymerase chain reaction

The polymerase chain reaction was done by maintaining the following proportion of reagents.

Table 3: List of reagents for the PCR.

Name	Quantity (uL)
Master mix	12.5
Forward primer	2
Reverse prime	2
DNA sample	2
Nuclease free water	6.5
Total	25

For the PCR amplification the universal 16s rRNA primer was used. The sequence of the forward primer is AGAGTTTGATCCTGGCTCAG and the sequence of the reverse primer is GGTTACCTTGTTACGACTT (Lane, 1991).

The PCR condition and timing were as follows.

Table 4: Duration and temperature for PCR.

Process	Temperature	Time
Initial denaturation	94	2 min
35 cycles		
Denaturation	94	30 sec
Annealing	52	30 sec
Extension	72	2 min
Final extension	72	5 min

2.11 Gel electrophoresis

The PCR products were further analysed through agarose gel electrophoresis. Here, 1% agarose gel in TE buffer was prepared. This was done by mixing 0.3g of agarose powder with 30ml of TE buffer. Ethidium bromide was used for gel staining. The PCR products were loaded into gel with 6x loading dye. To estimate size of template DNA, 1000bp DNA ladder was also loaded into the gel. The horizontal gel electrophoresis apparatus was used with TBE buffer as running buffer at 80V for 40 minutes. The results were observed using a UV transilluminator.

Chapter: 3

Results

3. Results

3.1 List of isolates

After plating on nutrient agar plates, distinct colonies of organisms were taken to be tested. A total of 30 isolates were taken.

The list of isolates is given below.

Table 5*: List of isolates*

Sample Number	Name of Isolates	Sample Number	Name of Isolates
1.	Bikrompur(1)	16.	Modhubon(2)
2.	Bikrompur(2)	17.	Modhubon(3)
3.	Bikrompur(3)	18.	Modhubon(4)
4.	Bikrompur(4)	19.	Muslim
5.	Vaggokul(1)	20.	Banoful(1)
6.	Vaggokul(2)	21.	Banoful(2)
7.	Vaggokul(3)	22.	Noni(1)
8.	Vaggokul(4)	23.	Noni(2)
9.	Jolkhabar(1)	24.	Ovijat(1)
10.	Jolkhabar(2)	25.	Ovijat(2)
11.	Bismillah(1)	26.	Captain's
12.	Bismillah(2)	27.	Prime(1)
14.	Alibaba(1)	28.	Prime(2)
14.	Alibaba(2)	29.	Rajvog(1)
15.	Modhubon(1)	30.	Rajvog(2)

A highest number of 4 isolates were found from Bikrampur, Bhaggokul and Modhuban. The lowest number was 1 and it was found from Captain's. The other shops were seemed to give 2 to 3 of isolates.

3.2 Findings of isolates found on selective media

After individual distinct colonies were marked, these were streaked on selective media. These media were Mannitol Salt Agar (MSA), Bacillus cereus Agar (BCA), Xylose Lysine Dextrose Agar, Eosine Methylene Blue Agar (EMB) and MRS agar.

The list of result from sub culture on selective media is given below

Table 6: Result of growth of the isolates in different selective media

Sample	BCA	MSA	XLD	MRSA	EMB	Probable Organism
Bikrompur(1)	+(Blue)	-	-	-	-	*Bacillus*
Bikrompur(2)	-	+ (yellow)	-	-	-	*Staphylococcus*
Bikrompur(3)	+(Blue)	+ (red colony)	-	-	-	*Bacillus*
Bikrompur(4)	-	-	-	-	-	n/a
Vaggokul(1)	-	+ (red)	-	-	-	*Staphylococcus*
Vaggokul(2)	-	+ (yellow)	-	-	-	*Staphylococcus*
Vaggokul(3)	-	+(yellow)	-	-	-	*Staphylococcus*
Vaggokul(4)	-	+ (yellow)	-	-	-	*Staphylococcus*
Jolkhabar(1)	-	+ (red)	-	-	-	*Staphulococcus*
Jolkhabar(2)	+(Blue)	+ (red)	-	-	-	*Bacillus*
Bismillah(1)	-	-	-	-	-	*Staphylococcus*
Bismillah(2)	+(Blue)	-	-	-	-	*Bacillus*
Alibaba(1)	-	+ (yellow)	-	-	-	*Staphylococcus*
Alibaba(2)	-	+ (yellow)	-	-	-	*Staphylococcus*
Modhubon(1)	+(Blue)	-	-	-	-	*Bacillus*
Modhubon(2)	+(Blue)	-	-	-	-	*Bacillus*
Modhubon(3)	+(Blue)	-	-	-	-	*Bacillus*
Modhubon(4)	+(Blue)	-	-	-	-	*Bacillus*
Muslim	+(blue)	-	-	-	-	*Bacillus*
Banoful(1)	-	+(yellow)	-	-	-	*Staphylococcus*
Banoful(2)	+(Blue)	-	-	-	-	*Bacillus*
Noni(1)	-	+(yellow)	-	-	-	*Staphylococcus*
Noni(2)	+(blue)	-	-	-	-	*Bacillus*
Ovijat(1)	+(Blue)	-	-	-	-	*Bacillus*
Ovijat(2)	+(Blue)	-	-	-	-	*Bacillus*
Captain's	+(blue)	-	-	-	-	*Bacillus*
Prime(1)	-	-	-	-	-	n/a
Prime(2)	-	+ (yellow Colony)	-	-	-	*Staphylococcus*
Rajvog(1)	-	+ (yellow)	-	-	-	*Staphylococcus*
Rajvog(2)	-	+ (yellow)	-	-	-	*Staphylococcus*

Among the 4 types of media that has been used, Mannitol Salt Agar showed the highest number of positive results, followed by Bacillus Cereus agar. The XLD and MRS agar showed no growth, indicating there was no prevalence of gram negative enteric bacteria and lactobacilli.

3.3 Findings from biochemical tests

After the subculture on selective media, the isolates were taken for biochemical tests. After growing in the selective media and taking for biochemical tests, 15 *Staphylococcus* isolates and 13 *Bacillus* isolates were found. Rest 2 did not show any specific result. All the samples were gram positive and they were either coccus or rod shaped. The ImVic test were done to see their potential to produce several organic compounds. This helped to different between the organisms, as the results are specific to the genus. All the samples were catalase and oxidase negative, which corresponds to the suspected genus they have been named as. All the isolates were also motile. Moreover, they were all negative in hydrogen sulphide production. The triple sugar iron test showed which sugars the organisms could ferment. This result varied in isolates. However, due to the presence of yellow butt in TSI tubes, it can be deduced that all the isolates were glucose fermenter.

The highest number of 11 organisms were found in Mohakhali. While the lowest number of 1 organism was found in Jahangir gate. Mohakhali, Uttara and Dhanmondi had the prevalence of 2 organisms, whereas, Jahangir Gate and Greenroad only had 1. The highest number of *S. aureus* was found in Mohakhali and highest number of *B. cereus* was found in Dhanmondi.

The results from the tests are given below.

Table 7: Result of biochemical tests done on the isolates

Sample number	MRVP		Catalase	Citrate	MIU			Gram stanining		TSI						Probable organism
	Methyl red	Vogesproskeur			Motility	Urease	Indole	Colour	Shape	Slant/butt colour	Glucose	Lactose	Sucrose	H_2S production	Gas production	
1. Bikrompur	+	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
2. Bikrompur	+	-	-	+	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
3. Bikrompur	-	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
4. Bikrompur	-	-	-	+	+	-	-	Purple	Rod	R/Y	+	-	-	-	-	*n/a*
5. Vaggokul	-	-	-	-	+	-	-	Purple	Cocci	R/Y	+	-	-	-	-	*Staphylococcus*
6. Vaggokul	-	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
7. Vaggokul	-	-	-	-	+	+	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
8. Vaggokul	-	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
9. Jolkhabar	+	-	-	-	+	-	-	Purple	Cocci	R/Y	+	-	-	-	-	*Staphylococcus*
10. Jolkhabar	-	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
11. Bismillah	+	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
12. Bismillah	+	-	-	-	+	-	-	Purple	Rod	R/Y	+	-	-	-	-	*Bacillus*
13. Alibaba	+	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
Sample n	MRVP		Ca	Cit	MIU			Gram stanining		TSI						Probable organism

	Methyl red	Vogesproskeur			Motility	Urease	Indole	Colour	Shape	Slant/butt colour	Glucose	Lactose	Sucrose	H_2S production	Gas production	
14. Alibaba	+	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
15. Modhubon	+	+	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
16. Modhubon	-	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
17. Modhubon	+	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
18. Modhubon	+	-	-	-	+	+	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
19. Muslim	+	-	-	-	+	+	-	Purple	Rod	R/Y	+	-	-	-	-	*Bacillus*
20. Banoful	+	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
21. Banoful	+	-	-	-	+	-	-	Purple	Rod	R/Y	+	-	-	-	-	*Bacillus*
22. Noni	+	-	-	-	+	-	-	Purple	Cocci	R/Y	+	-	-	-	-	*Staphylococcus*
23. Noni	+	-	-	-	+	-	-	Purple	Rod	R/Y	+	-	-	-	-	*Bacillus*
24. Ovijat	-	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
25. Ovijat	-	+	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
26. Captain's	-	-	-	-	+	-	-	Purple	Rod	Y/Y	+	+	+	-	-	*Bacillus*
27. Prime	Neutral	-	-	-	+	+	-	Purple	Cocci	Y/Y	+	+	+	-	-	*n/a*
28. Prime	Neutral	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Stahphylococcus*
29. Rajvog	+	-	-	-	+	-	-	Purple	Cocci	Y/Y	+	+	+	-	-	*Staphylococcus*
30. Rajvog	+	-	-	-	+	-	-	Purple	Cocci	R/Y	+	-	-	-	-	*Staphylococcus*

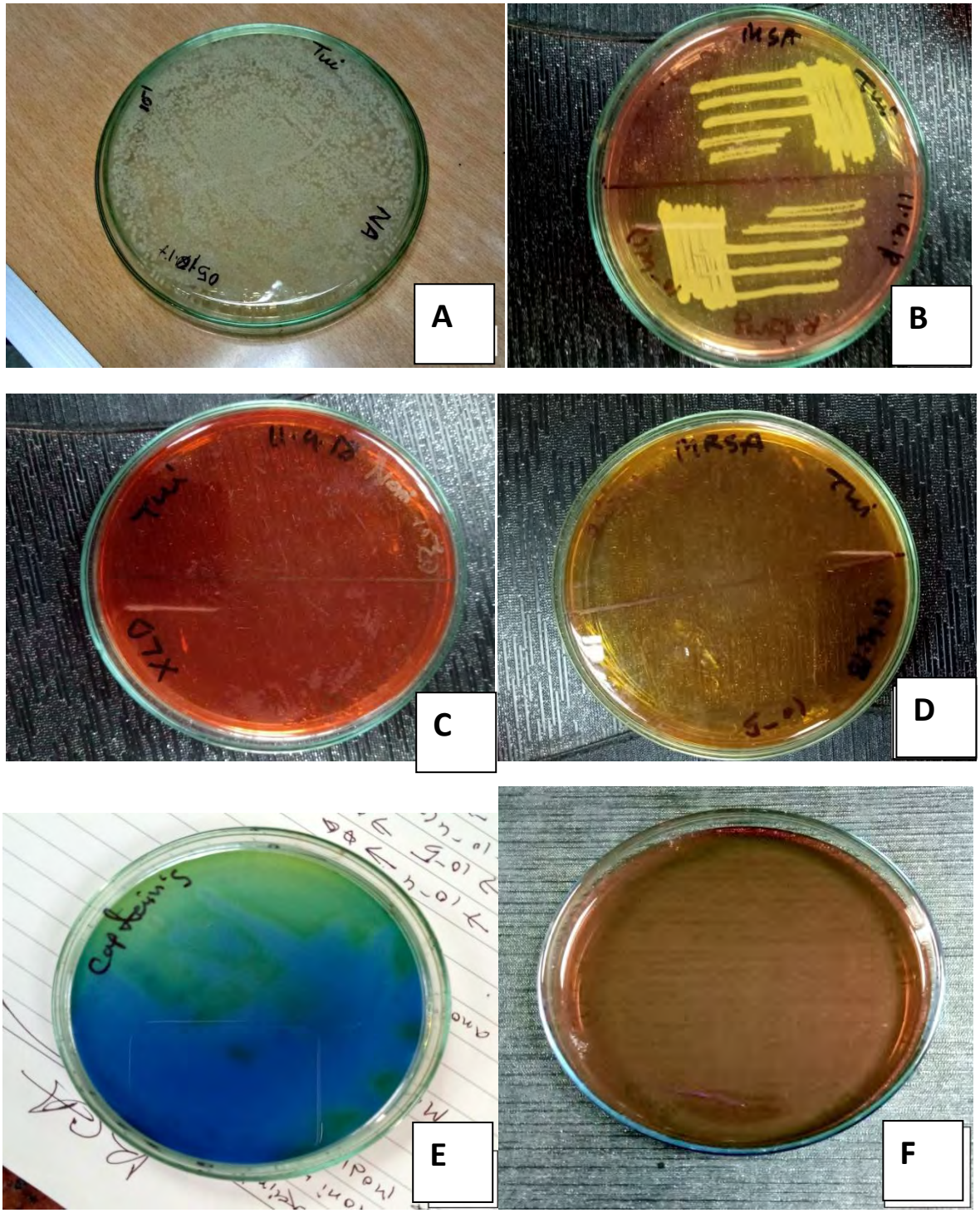

Figure A : Suspension spread on NA showing distinct colonies ; Figure B : Isolate streaked on MSA;Figure C : Isolates streaked on XLD ; Figure D : Isolates streaked on MRS agar ; Figure E : Isolates streaked on BCA ; Figure F : Isolates streaked on EMB

The number and percentage of isolates are given below

Table 4: Percentage of organisms

Organism name	Number	Percentage (%)
Staphylococcus	15	50
Bacillus	13	43.3
Unidentified	2	6.7

In terms of the area of the origin of the isolates, the results were as follows.

Table 5: Number of isolates in terms of area

	Mohakhali	Uttara	Greenroad	Dhanmondi	Jahangirgate
Staphylococcus	8	4	-	1	-
Bacillus	3	3	2	6	1
Total	11	7	2	7	1

Among the antibiotics used, ciprofloxacin is the most effective one as 100% of the isolates were sensitive to it. The second highest was gentamicin with only 6.67% being resistant to it. The least effective antibiotic was cefepime with having 100% of the isolates being resistant to it followed by cephalexin to which 93.33% isolates were resistant.

The two isolates from Captain's and Modhubon showed highest resistance for the antibiotics used. These two were *Bacillus* species. Later, these two samples were taken for DNA extraction and PCR.

3.4 Antibiotic sensitivity test

Table 6: Result of antibiotic sensitivity testing done on the isolates

Serial	Antibiotics																					
	NOR		COT		AMX		VA		TM		CL		CTR		CFM		CXM		AZM		GEN		CIP	
	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int
1	22	S	20	S	0	R	15	R	22	S	12	R	22	S	12	R	22	S	32	S	22	S	32	S
2	0	R	0	R	0	R	0	R	R	R	R	R	20	S	15	R	0	R	25	S	0	R	30	S
3	20	S	28	S	0	R	15	R	20	S	8	R	20	S	0	R	24	S	22	S	25	S	28	S
4	20	S	22	S	0	R	12	R	20	S	10	R	22	S	0	R	25	S	22	S	23	S	29	S
5	19	S	0	R	0	R	16	R	0	R	0	R	18	S	0	R	10	R	27	S	20	S	25	S
6	24	S	18	S	0	R	16	R	0	R	0	R	12	R	0	R	0	R	30	S	25	S	29	S
7	25	S	0	R	0	R	10	R	0	R	0	R	14	R	0	R	0	R	25	S	24	S	29	S
8	25	S	0	R	0	R	17	R	0	R	0	R	15	R	0	R	0	R	25	S	24	S	30	S
9	18	S	26	S	16	R	16	R	19	S	9	R	20	S	0	R	9	R	22	S	22	S	21	S
10	26	S	0	R	15	R	19	S	0	R	9	R	20	S	0	R	0	R	29	S	23	S	28	S
11	24	S	0	R	13	R	10	R	0	R	10	R	19	S	0	R	0	R	29	S	24	S	29	S
12	24	S	12	R	0	R	9	R	0	R	0	R	0	R	14	R	0	R	31	S	24	S	32	S
13	22	S	0	R	0	R	0	R	0	R	0	R	18	S	R	R	0	R	30	S	23	S	30	S
14	27	S	28	S	10	R	17	R	17	R	28	S	15	R	17	R	12	R	28	S	24	S	33	S
15	24	S	16	S	0	R	17	R	0	R	0	R	19	S	R	R	0	R	23	S	24	S	29	S

S=Sensitive R=Resistant

Table 6: Result of antibiotic sensitivity testing done on the isolates

Serial	Antibiotics																					
	NOR		COT		AMX		VA		TM		CL		CTR		CFM		CXM		AZM		GEN		CIP	
	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int	ZS	Int
16	30	S	27	S	28	S	19	S	10	R	12	R	27	S	R	R	15	R	29	S	27	S	36	S
17	16	S	30	S	29	S	19	S	10	R	12	R	26	S	8	R	9	R	29	S	29	S	34	S
18	22	S	0	R	0	R	16	R	0	R	0	R	9	R	0	R	0	R	28	S	10	R	30	S
19	25	S	0	R	0	R	17	R	0	R	0	R	17	S	0	R	0	R	21	S	21	S	29	S
20	24	S	26	S	25	S	13	R	13	R	14	R	20	S	0	R	0	R	26	S	22	S	30	S
21	25	S	0	R	0	R	19	S	0	R	0	R	15	R	0	R	0	R	22	S	22	S	27	S
22	20	S	30	S	19	S	18	S	22	S	10	R	23	S	0	R	0	R	22	S	25	S	29	S
23	21	S	30	S	20	S	17	S	21	S	10	R	25	S	10	R	13	R	29	S	27	S	30	S
24	29	S	26	S	26	S	19	S	13	S	15	R	23	S	0	R	0	R	27	S	25	S	36	S
25	25	S	28	S	19	S	17	S	20	S	10	R	27	S	0	R	12	R	27	S	25	S	30	S
26	30	S	29	S	21	S	20	S	14	S	14	R	23	S	0	R	0	R	29	S	25	S	36	S
27	30	S	26	S	25	S	20	S	0	R	14	R	24	S	0	R	10	R	26	S	25	S	38	S
28	17	R	0	R	0	R	0	R	0	R	10	R	10	R	0	R	0	R	24	S	20	S	30	S
29	10	R	0	R	0	R	0	R	0	R	15	R	0	R	0	R	0	R	20	S	20	S	27	S
30	10	R	0	R	0	R	0	R	0	R	15	R	0	R	0	R	0	R	0	R	18	S	30	S

S=Sensitive R=Resistant

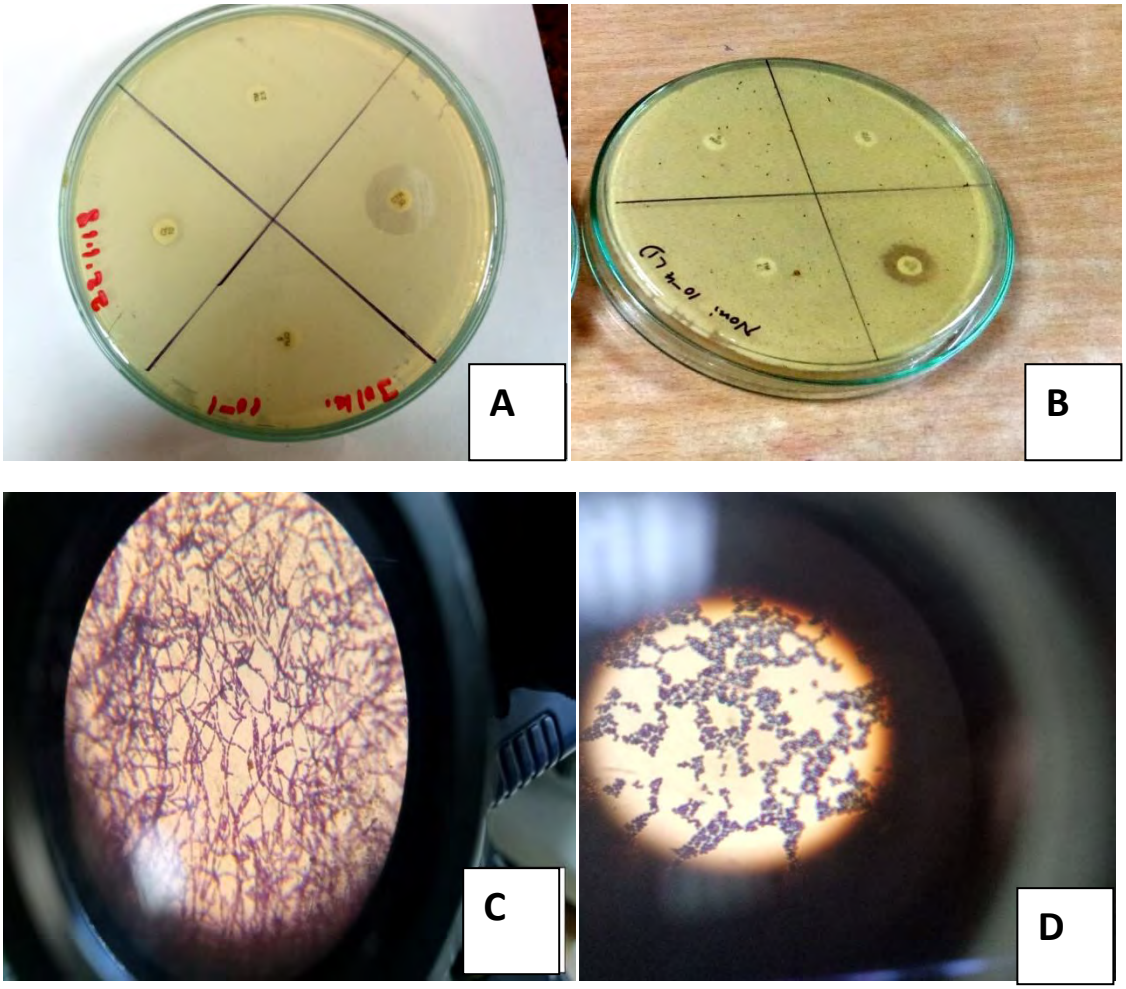

Figure A: Antibiogram test; Figure B: Antibiogram test ; Figure C : Microscopic observation of gram positive rod shaped bacteria ; Figure D : Microscopic observation of gram positive cocci bacteria

3.5 Polymerase chain reaction and electrophoresis findings

Two samples-Captain's and Modhubon's which were suspected to be *Bacillus* spp and showed highest antibiotic resistance were taken for polymerase chain reaction after DNA extraction. After being run in gel electrophoresis, the both samples gave distinct bands at approximately at around 1500bp (Alshaikh*et al.*, 2017).

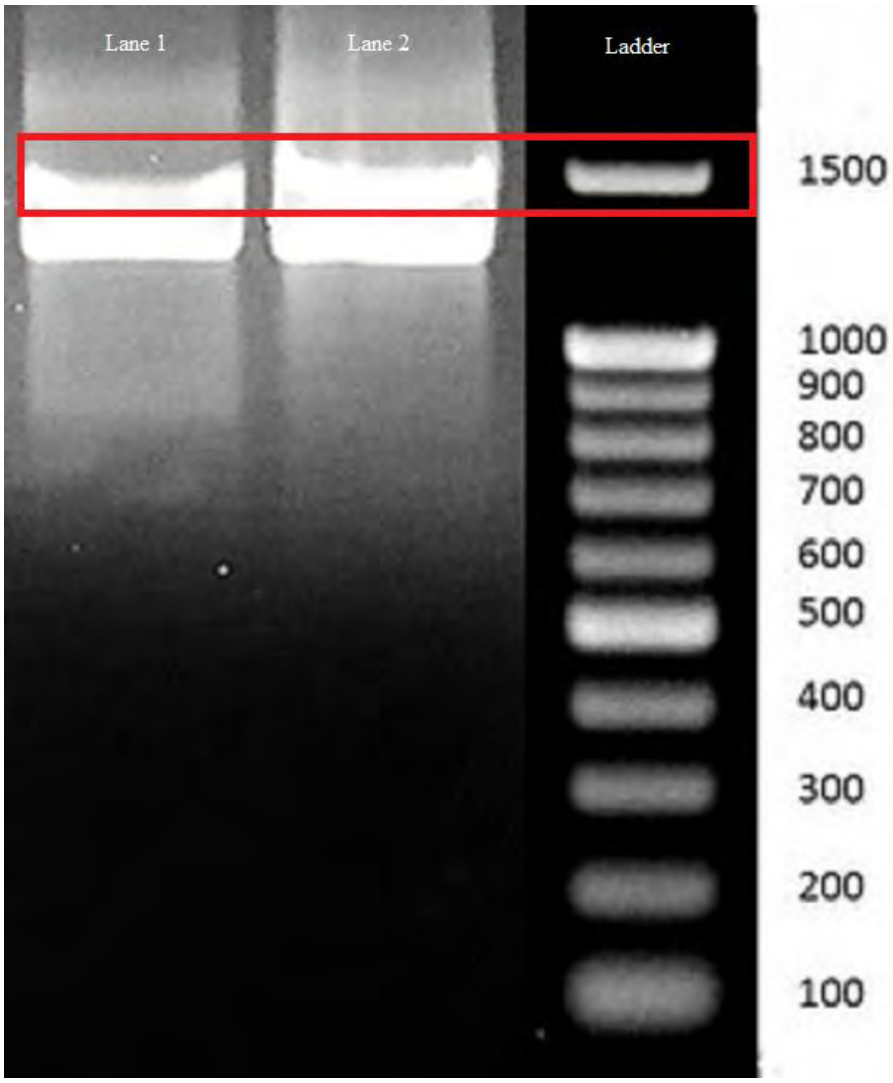

Figure: Gel Electrophoresis Result

For the clearer species level of identification, 16s rRNA is the most effective one. The samples which were suspected to be *Bacillus* were taken for PCR with 16s rRNA primers. This section of gene was chosen due to their very slow mutation rate and specificity to species level. In other studies, 16s rRNA primers were used to identify different *Bacillus* species. Two samples from the 13 *Bacillus* isolates were taken. After the PCR, the both samples gave band at about 1500 bp, which corresponds to their band size for 16s rRNA amplification. Similar band size

was seen for another study involving child food. This shows the effectivity of the PCR method for rapidly detecting *Bacillus* from food samples. Moreover, for construction of phylogenetic tree to compare evolutionary relationship, 16s rRNA sequence is considered highly effective. Additionally, after sequencing the 16s rRNA region of the *Bacillus* samples, similar phylogenetic analysis will be done.

Chapter: 4
Discussion

4. Discussion

The purpose of the study was to find out microorganisms in Swandesh that can cause potential health hazards. There have been many studies on other dairy products such as yogurt, sweetmeats, cottage cheese and so on. These included searching for *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella* spp, *Lactobacillus*spp and so on. While the organisms like *E. coli* and *Salmonella* are enteric bacteria, gram positive *S. aureus* or *B. cereus* are found on the skin and soil. These organisms can cause massive health damage that can vary from toxic shock syndrome to diarrhoea (Masud*et al*., 1989). In previous studies done on milk samples, yogurt and cottage cheese sweetmeat, high level of microbial contamination was seen (Rahman *et al*., 2015). However, there were no research work done on Swandesh in Bangladesh. Swandesh is regularly consumed and very popular in this country. A study on the microbial contamination of Swandesh was highly important.

Milk based desserts are very common in this subcontinent. Due to their high level of nutrition, they harbour many pathogenic bacteria. In this study, Swandesh was checked from the presence of harmful bacteria. From various locations of Dhaka city, 14 samples were taken and of which 30 organisms were isolated. All the isolates were gram positive. This corresponds to a study by Alnakip (2009), where he found only gram positive bacteria. Dairy products like Swandesh go through cooking at a high temperature. Additionally, these products have high osmotic pressure. Gram negative bacteria with very thin to low level of peptidoglycan layer, cannot tolerate high osmotic pressure (Mille *et al*., 2005). As a result, in cooked food like dairy products, they are often not found. From the study, it was seen, 50% of the isolates were *Staphylococcus* and 43.3% were *Bacillus.* In a study from 1991-1994, it was seen that 3% of the isolated microorganism from food were *Bacillus* (Hatakka, 2004).Additionally, according to European food safety (2007), 17.1% of isolates in food poisoning were by *Bacillus* species. Moreover, a major source of contamination in sweetmeats is hands of the maker. In salty skin condition, halophilic organisms such as *Bacillus* and *Staphylococcus* may survive. This explains the result of having more than 50% *Staphylococcus* and the rest as *Bacillus*. Although, in other studies done before, the percentage of *Staphylococcus* ranged from 0% to 12% (Singh & Prakash, 2008; Normanno *et al.*, 2007). This high difference between previous study and the current one could be explained by the fact that those involved dairy products that did not involve extensive hand use as the process of Swandesh. Swandesh preparation takes direct contact of hands in the shaping. This can explain high level of *Staphylococcus*.

Even though two firmicutes *Bacillus* and *Staphylococcus* were present in the samples, one other common firmicute that is predominant in dairy product, *Lactobacillus* was absent from the samples. This is opposite of the result found from a study by Harun-ur-Rashid *et al*. (2007). Although, in another study by Rahman *et al* (2008), they found pathogenic enteric bacteria in Kacha gollabut no *Staphylococcus aureus.* In other similar studies with dairy products, *Lactobacillus* was highly predominant in fermented dairy products (Shangpliang*et al*., 2018).

However, after streaking the isolates in MRS agar, none of the isolates gave positive result in it. One possible reason could be that Swandesh has very low moisture. At this low level of moisture bacteria often go to dormant phase. In a previous study by Troller and Stionson (1981), it was seen that low level of moisture inhibits *Lactobacillus* activity.

Among the 30 samples, 2 were not identified. The study involved identification of selected pathogenic organisms such as *Staphylococcus, Bacillus, Salmonella, Shigella* and *Escherichia coli*. The isolates were then streaked onto selective media for those specific organisms only. One of those two unidentified isolates was coccus, and another was rod. Our skin harbours many gram-positive bacteria as normal skin flora. These involve rod shaped coryneform, *Acinetobacter* species and many more along with cocci such *Streptococcus* species (Davis, 1996).

The study also looked at the demographic distribution of the organisms found. It was seen that Mohakhali and Dhanmondi area had the highest number of isolates. Mohakhali had the highest number of *Staphylococcus*, it shows a concerning issue regarding the personal hygiene of the workers in the shop. While, Dhanmondi area had the highest number of *Bacillus*. *Bacillus* is often found in milk. It comes from the soil or the farm where milk is harvested (Christiansson *et al.*, 1999). A high number of the presence of *Bacillus* indicates presence of contamination in milk source. While, no other organisms were found because milk was boiled during preparation. Enteric organisms like *Salmonella* and *E coli* are usually heat labile. In a study by Mattic *et al.* (2001) it was seen that at temperature above 65ºC enteric organisms were unable to grow.

After isolation and screening, the antibiotic sensitivity pattern was also checked. All the isolates were sensitive to cefixime (100%), while all the samples were sensitive to ciprofloxacin. Only 8% of the samples were resistant to gentamicin, which corresponds to another study of *Bacillus* samples (Barbosa *et al.*, 2005). In terms of organism, the *Bacillus* isolates were most resistant. Their resistance can be explained by their thick cell wall that prohibits antibiotics from working inside.

After reviewing the antibiotic sensitivity test results, the *Bacillus* isolates were taken for molecular level identification. It was done to compare the effectiveness of the PCR method with traditional biochemical tests. Moreover, *Bacillus* suspected organisms were taken for molecular analysis due their high virulence level (Beecher *et al*., 1995). Furthermore, *Bacillus* was chosen instead of *Staphylococcus* isolates, because of the difficulty of identifying *Bacillus* species. Whereas, *Staphylococcus* has only 10 species and can be distinguished by biochemical tests (Kateete, *et al*., 2010), *Bacillus* has numerous species and cannot be differentiated easily (Tuazon, 2010). For the clearer species level of identification, 16s rRNA is the most effective one (Ash *et al*., 1991). The samples which were suspected to be *Bacillus* were taken for PCR with 16s rRNA primers. This section of gene was chosen due to their very slow mutation rate and specificity to species level. In other studies, 16s rRNA primers were used to identify different *Bacillus* species (Sacchi*et al.,* 2002). Two samples from the 13 *Bacillus* isolates were taken. After the PCR, the both samples gave band at approximately at around 1500 bp, which

corresponds to their band size for 16s rRNA amplification (Reza *et al.*, 2016). Similar band size was seen for another study involving child food (Alshaikh*et al.*, 2017). This shows the effectivity of the PCR method for rapidly detecting *Bacillus* from food samples. Moreover, for construction of phylogenetic tree to compare evolutionary relationship, 16s rRNA sequence is considered highly effective (Stackebrandt & Goebel, 1994).

This study gives a warning about the hygiene scenario of dairy product industry. The presence of microbes like *Staphylococcus* and *Bacillus* indicated improper hygiene and imminent health issues. Especially, as these products are regularly consumed by children, the risk gets even higher. Moreover, some of the samples were resistant to several antibiotics. This increases the concern as the treatment becomes harder. The outcome of the study could be publicized for creating awareness to eradicate improper hygiene practice. Moreover, the presence of antibiotic resistant genes can be checked by PCR method for the isolated samples. Additionally, samples from different places of the country could be evaluated to see a pattern of microorganisms

Further construction of phylogenetic tree can be useful for identifying the coding sequences of *Bacillus* spp. A phylogram can be constructed to differentiate between *Bacillus* and other bacterial species. The coding DNA sequences can be taken from the NCBI and we can relate the *Bacillus* species with the other groups of bacteria. The ancestors of this group of bacteria and their lineage group can also be identified. (Raza *et al*., 2016)

5. References

Alnakip, Mohamed. (2009). Prevalence of gram positive bacteria in milk and some dairy products. MV Sc.

Alshaikh, KS.,Mutwakil. MH.,& Ahmed, MMM. 2017. Isolation and Identification of Bacillus cereus from Infant Food Using 16s rRNA Sequence. *Advances in Biological Research*

Amani, J., Mirhosseini, S. A., & Imani Fooladi, A. A. (2015). A Review Approaches to Identify Enteric Bacterial Pathogens. *Jundishapur Journal of Microbiology*. http://doi.org/10.5812/jjm.17473

Ash, C., Farrow, J. ,Wallbanks, S. and Collins, M. (1991), Phylogenetic heterogeneity of the genus *Bacillus*revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Letters in Applied Microbiology*

Barbosa TM, Serra CR, La Ragione RM, Woodward MJ, &Henriques AO. 2005. Screening for Bacillus isolates in the broiler gastrointestinal tract. *Appl. Environ. Microbiol*

Beecher DJ, Pulido JS, Barney NP &Wong AC. 1995. Extracellular virulence factors in *Bacillus* cereus endophthalmitis: methods and implication of involvement of hemolysin BL. *Infect Immun*.

Checkley, W., Buckley, G., Gilman, RH., Assis, AM., Guerrant, RL., Morris, SS. et al. 2008. Multi-country analysis of the effects of diarrhoea on childhood stunting. *Int J Epidemiol.*

Christiansson, A., Bertilsson, J. &Svensson, B. 1999. Bacillus cereus spores in raw milk: factors affecting the contamination of milk during the grazing period.*J Dairy Sci.*

Cogen, A. L., Nizet, V., & Gallo, R. L. (2008). Skin microbiota: a source of disease or defence? *The British Journal of Dermatology*, *158*(3), 442–455. http://doi.org/10.1111/j.1365-2133.2008.08437.x

Davis CP. Normal Flora. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 6. Available from: https://www.ncbi.nlm.nih.gov/books/NBK7617/

Evans Jr., Doyle J.&Evans, DG. 2007. "Escherichia Coli". Medical Microbiology, 4th edition. The University of Texas Medical Branch at Galveston

Green, D. W. (2002). The bacterial cell wall as a source of antibacterial targets. *Expert Opinion on Therapeutic Targets*, *6*(1), 1–20. https://doi.org/10.1517/14728222.6.1.1

Harun-ur-Rashid, M., Togo, K., Useda, M. & Miyamoto, T. 2007. Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk “Dahi” in Bangladesh. *Pakistan J. Nutr.*

Hatakka M (1998) Microbiological quality of hot meals served by airlines.*J Food Prot*

Kateete, DP., Kimani, CN., Katabazi, F A., Okeng, A., Okee, M S., Nanteza, A., Joloba, ML &Najjuka, FC. 2010. Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials*

Khan I&Kang SC. 2016. Probiotic potential of nutritionally improved *Lactobacillus plantarum*DGK-17 isolated from kimchi -A traditional Korean fermented food. Food Control

Lane, D.J. 1991. 16S/23S rRNAsequencing.*Nucleic acid techniques in bacterial systematics* (pp. 115–175).

Larsen, H. D., &Jourgeusen, K. 1996. Occurrence of *Bacillus cereus* in Danish Pasteurized milk. *International J of Micro*

Logan NA. (2011). Bacillus and relatives in foodborne illness. *J ApplMicrobiol*

Mattick, K. L., Jørgensen, F., Wang, P., Pound, J., Vandeven, M. H., Ward, L. R., … Humphrey, T. J. (2001). Effect of Challenge Temperature and Solute Type on Heat Tolerance of *Salmonella* Serovars at Low Water Activity. *Applied and Environmental Microbiology*, *67*(9)

Mille, Y., Beney, L.,& Gervais, P. (2005). Compared tolerance to osmotic stress in various microorganisms: Towards a survival prediction test. Biotechnology and bioengineering. 92. 479-84. 10.1002/bit.20631.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., &Erlich, H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Paper presented at: Cold Spring Harbor symposia on quantitative biology (Cold Spring Harbor Laboratory Press)

Nikaido, H. (2010). Multidrug Resistance in Bacteria. *Annu Rev Biochem.*, (2), 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923.Multidrug

Normanno, G., La Salandra, G., Dambrosio, A., Quaglia, N.C., Corrente, M., Parisi, A., Santagada, G., Firinu, A., Crisetti, E. and Celano, G.V. (2007). Occurrence, characterization and antimicrobial resistance of entero- toxigenic Staphylococcus aureus isolated from meat and dairy products. Int. J. Food Microbiol.

Ogier, J.-C., Son, O., Gruss, A., Tailliez, P., & Delacroix-Buchet, A. (2002). Identification of the Bacterial Microflora in Dairy Products by Temporal Temperature Gradient Gel Electrophoresis. *Applied and Environmental Microbiology.*

Oliver, SP., Jayaro, BM., & Almeida RA. 2005. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications.*FoodbornePathog Dis.*

Pal, M., Mulu, S., Tekle, M., Pinto, S., &Prajapati, J P. (2016). Bacterial Contamination of Dairy Products. *Beverage and Food World*.

Payne, D. N., & Wood, J. M. 1974. The incidence of enterotoxin production from *Staphylococcus aureus* strains isolated from food. *Applied J of Bacteriology*.

Pawlowski, SW., Warren, CA.,&Guerrant, R. 2009. Diagnosis and treatment of acute or persistent diarrhea. *Gastroenterology*.

Presscott, L.M., Harley, J.P. and Klein, D.A. (2002). Textbook of Microbiology. Brown Publishers. 5th ed.: 441-442.

Rahman, M.M., Mashiar, RM., S. M. Arafat, Rahman, A., Khan, M. Z. H., & Rahman, MS. 2008. Microbiological Quality Assessment of a Local Milk Product, Kwacha Golla, of Bangladesh. *J. Appl. Biol. Chem.*

Rahman, T., Akon,T., Sheuli, IN &Hoque, N. 2015. Microbiological analysis of raw milk, pasteurized milk and yogurt samples collected from different areas of Dhaka city, Bangladesh. *Journal of Bangladesh Academy of Sciences*.

Raza, Shahid& Ameen, Ayesha. (2016). Comparison of 16S rRNA Gene of Bacillus cereus with Different Bacterial Species. Journal of Advances in Biology & Biotechnology. 7. 1-6. 10.9734/JABB/2016/26524.

Reid G., Cook R. L., &Bruce A. W. (1987) Examination of strains of lactobacilli for properties which may influence bacterial interference in the urinary tract. *J. Urol.*

Reller, LB., Weinstein, M., Jorgensen, JH., Ferraro, MJ. 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices, *Clinical Infectious Diseases*

Sacchi, C. T., Whitney, A. M., Mayer, L. W., Morey, R., Steigerwalt, A., Boras, A., Popovic, T. (2002). Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of *Bacillus anthracis*. *Emerging Infectious Diseases*, *8*(10), 1117–1123. http://doi.org/10.3201/eid0810.020391

Salminen S., Isolauri E., Salminen E. (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Leeuwenhoek.

Sen, D. C. &Rajorhia, S. G. (1989) Quality characteristics of Sandeshmarketed in Calcutta. Indian J Dairy Sci 42:241–245

Shangpliang, H N J., Rai, R., Keisam, S., Jeyaram, K., &Tamang, J P. 2018. Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing. *Scientific reports*

Singh, P. and Prakash, A. (2008). Isolation of Escherichia coli, Staphylococcus aureus and Listeria monocytogenes from milk products sold under market conditions at Agra Region. Acta Agri. Slov.

Soomro, A.H., Arain, M.A., Khaskheli, M. and Bhutto, B. 2003. Isolation of Staphylococcus aureus from milk products sold at sweet meat shops of Hyderabad. *Online J. Biol. Sci.*, 3(1): 91-94.

Stackebrandt, E., & Goebel, B. M. 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of systemic and evolutionary microbiology.*

Su LH, Chiu CH. (2007). "Salmonella: clinical importance and evolution of nomenclature". *Chang Gung Medical Journal*

Swaminathan B, Ayres JC, Williams JE. 1978. Control of nonspecific staining in the fluorescent antibody technique for the detection of salmonellae in foods. Appl Environ Microbiol.

Tamang, J. P., Watanabe, K. &Holzapfel, W. H. 2016 Review: Diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol*

Tortora, G. J., Funke, B. R. & Case, C. L. (2010). *Microbiology: An introduction*.

Troller, J A. & Stinson, J V. 1981. Moisture Requirements for Growth and MetaboliteProduction by Lactic Acid Bacteria. *Applied and environmental microbiology.*

Tuazon, C U. 2010. *Bacillus* species. *Journal of infectious disease.*