Development of Lactic Acid Bacteria Starter Culture for Probiotic Yogurt

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

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Dedicated to

My Beloved Parents and Lovely Sister

Declaration

I hereby declare that this research work entitled "**Development of Lactic Acid Bacteria Starter Culture for Probiotic Yogurt**" presented in it was carried out and submitted by me, Tamannyat Binte Eshaque to the Department of Mathematics and Natural Sciences under the supervision and guidance of Dr. M. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University. This thesis work has been generated as the result of my own original research and submitted in the partial fulfillment for the degree of Bachelors of Science in Biotechnology in BRAC University. I would also like to declare that, this presented work is original and has not been submitted elsewhere for any degree or diploma. The other books, articles and websites, which I have made use of are acknowledged at the respective place in the text.

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Abstract

Lactobacillales or lactic acid bacteria (LAB) are one of the most numerous groups of bacteria linked to humans. Lactic acid bacteria show antimicrobial properties and these are generally recognized as safe (GRAS). The primary antimicrobial effect exhibited by the LAB is the production of lactic acid and a lower pH. In recent years, it has been seen that, several pathogenic bacteria are getting resistant to multiple antibiotics. In such conditions, new probiotics with potential antimicrobial properties can play an important role. In the present study, the industrially important lactic acid bacteria are mainly focused with the purpose of creating a probiotic starter culture. The study started with 20 isolates of LAB isolated from fermented vegetables and dairy products from the previous project. Among them 10 isolates were selected with maximum growth on MRS media. The antagonistic activity of these isolates was monitored using several parameters. The agar well diffusion results zone of inhibition with the cell-free supernatant of LAB isolates derived from Cauliflower, Carrot, Cabbage, Brinjal, Aarong yogurt, Dhaka cheese against indicator organisms *Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus pneumoniae, Bacillus subtilis, Bacillus cereus, Shigella dysenteriae, Shigella flexneri, Klebsiella sp., Enterobacter cloacae,* and *Salmonella typhi*. Measured pH of cell-free supernatant and MRS broth was in higher range.

The aim of this study is to find out antimicrobial properties present in LAB strains isolated from fermented vegetables and dairy products and developing an economically affordable probiotic yogurt starter culture. The production of probiotic yogurt starter culture involved inoculating three types of milk: whole milk, skimmed milk and powdered milk with the LAB isolates. Four hours to an overnight incubation at 30-40 ºC showed desired yogurt production. Further dehydration of a portion of the yogurt at 65 ºC for 2-3 hours gave yogurt powder. Finally addition of the produced yogurt powder into milk sample resulted in yogurt again. Such yogurt powder can be industrially manufactured at a low cost in developing countries where the consumption of probiotic products is growing intensively. Consumption of active probiotic yogurt can eventually lessen the intake of traditional antibiotics.

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WHO: World Health Organization

LAB: Lactic Acid Bacteria

GRAS: Generally Recognized as Safe

BPB: Bacteriocin Producing Bacteria

BLS: Bacteriocin-like substance

MRS: de Man, Rogosa and Sharpe

NA: Nutrient Agar

MH: Mueller Hinton

ZOI: Zone Of Inhibition

ºC: Degree Celsius

CFS: Cell free supernatant

rpm: Rotation per minute

GIT: Gastrointestinal tract

***et al*:** and others

spp.: Species

kDa: Kilo Dalton

gm: Gram

ml: Milliliter

µl: Microliter

CFU: Colony Forming Unit

Conc.: Concentration

H_2O_2: Hydrogen peroxide

Chapter 1:
Introduction

1. Introduction

1.1 Background:

In recent days, food is not considered on the basis of taste only, but it must have immediate nutritional value and health benefits. The food industries around the world are busy with competition to increase the nutritional value of their marketed food products. This increase in nutrition in today's concept is obtained by the consumption of food containing live bacteria and from this, the idea of 'Probiotics' stepped in. In the concept of functional food, especially in the dairy industry, there is an increasing interest in probiotic products that contain lactic acid bacteria of intestinal origin (J. Suskovic et al., 2010). During the last decades, it became clear that the human body lives in close harmony with a complex ecosystem that is composed of more than 1000 different bacterial species inhabiting the oral cavity, upper respiratory tract, gastrointestinal tract (GIT), vagina and skin. This collection known as the 'microbiota' is acquired soon after birth and persists throughout life. Together, these microbes play an important role in the physiology of their host, including the digestion and assimilation of nutrients, protection against pathogen colonization, modulation of immune responses, regulation of the fat storage, and stimulation of intestinal angiogenesis (S. Pithva et al., 2011). If these classes of microbes are ingested regularly, it can become a good alternative to many antibiotics as there has been a rapid worldwide increase in multi-drug resistant pathogenic bacteria that are resistant to multiple antibiotics.

There are a large number of probiotic foods back from the ancient times which are mostly originated from fermented foods like fermented vegetables as well as cultured dairy products. The quest to find food ingredients with valuable bioactive properties has encouraged interest in lactic acid bacteria (LAB) with probiotic attributes such as antimicrobial activity against pathogenic microorganisms, antiviral activity, anti-yeast property, anti-mutagenic, anti-platelet aggregation and antioxidant attributes (Kazemipoor et al., 2012). This study is focused on the antimicrobial properties of LAB isolated from fermented foods and from that it will target to establish a probiotic product with nutritional value and future health benefits.

1.2 Lactic acid bacteria (LAB):

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, catalase negative cocci or rods which produce lactic acid a major end product from the fermentation of carbohydrates (Darsanaki et al., 2012). In the first case, two molecules of lactate are generated and in the second, lactate, ethanol and carbon dioxide are produced. Lactic acid bacteria are also able to produce small organic substances that contribute with aroma and give specific organoleptic attributes to the final products (Caplice and Fitzgerald 1999). Lactic acid bacteria include various major genera: *Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus,* and *Weissella*. Other genera are *Aerococcus*, *Microbacterium*, *Propionibacterium,* and *Bifidobacterium* (Carr et al., 2002). *Lactobacillus acidophilus*, *L. plantarum*, *L. Casei*, *L. rhamnosus*, *L. delbrueckii bulgaricus*, *L. fermentum*, *L. reuteri*, *Lactococcus lactis lactis*, *Lactococcus lactis cremoris*, *Bifidobacterium bifidum*, *B. infantis*, *B. adolecentis*, *B. longum*, *B. breve*, *Enterococcus faecalis*, *Enterococcus faecium*, are some of the most common species (Garrity 1984; Dellaglio et al. 1994), and some strains are recognized as probiotics (Fuller, 1989; Parada et al., 2003).

1.2.1 Properties and characteristics:

They contain a low GC content and are acid-tolerant (able to grow at pH 4.4). These organisms are generally non-sporulating and non-respiring rod or cocci. Taxonomically, the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales*, family *Lactobacillaceae* (S. Pithva et al., 2011). Cells are non-motile, irregular rods with rounded or often tapered ends (0.5-1.0 or 1.5-2.0μm) occurring singly, in pairs, or irregular short chains, often forming clumps. It belongs to biohazard group I. The G·C content (mol %) is 45 and the lactic acid isomer is DL (Lavanya et al., 2013). They are nutritionally fastidious, requiring rich media to grow (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins) (S. Pithva et al., 2011).

1.2.2 Sources of Lactic acid bacteria:

LABs are widely distributed in nature, they are typically involved in a large number of the spontaneous food fermentation (Mezaini et al., 2009). They are naturally associated with mucosal surfaces, particularly the gastrointestinal tract, and are also indigenous to food-related habitats, including plant (fruits, vegetables, and cereal grains), wine, milk, and meat environments. The LAB includes both important pathogens, e.g., several *Streptococcus* species, and extremely valuable nonpathogenic species that are used for industrial fermentation of dairy products, meats, and vegetables, and they are also critical for the production of wine, coffee, silage, cocoa, and sourdough (Makarova & Koonin, 2007).

1.2.3 Kinds of Lactic acid bacteria:

Among LAB members, the genus *Lactobacillus* contains over 110 species which are classified in three major groups: the obligate homo or fermentative lactobacilli which ferment hexoses to lactic acid; the facultative heterofermentative lactobacilli, which ferment hexoses to lactic acid only or to lactic acid together with acetic acid, ethanol and formic acid under glucose limitation; and obligate heterofermentative lactobacilli, which ferment hexoses to lactic acid, acetic acid, ethanol and CO and ferment pentoses to lactic acetic acid (Darsanaki et al., 2012). Currently, 19 complete genomes of *streptococci* are available, covering different strains of five species (Makarova et al., 2007). There is a need for more studies on the genetic basis for probiotic properties which will give further understanding regarding novel manipulation skills and applicability in nutrition and health sectors.

1.2.4 Function and importance of Lactic acid bacteria:

Members of the LAB produce bacteriocins and bacteriocins-like substances (BLS) which may inhibit the growth of spoilage and pathogenic microorganisms. These properties of LAB can be used as bio-preservatives of foods using bacteriocinogenic lactic acid bacteria isolated directly from foods which can be an innovative approach. The preservative effect exerted by the LAB is mainly due to the production of organic acids (such as lactic acid) which result in lowered pH (Daeschel, 1989). LAB also produce antimicrobial compounds including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas et al., 2001). These microorganisms are found in milk, meat and, fermented products, as well as in

fermented vegetables and beverages, inhibiting the growth of pathogenic and deteriorating microorganisms, maintaining the nutritive quality and improving the shelf life of foods (Djadouni & Kihal, 2012).

1.3 Antimicrobial properties of Lactic acid bacteria:

Recent research has increasingly demonstrated the role of antimicrobial compounds as a protective mechanism against intestinal pathogens and therefore certain strains could have an effect on both the food and the gut (Arques et al., 2015).

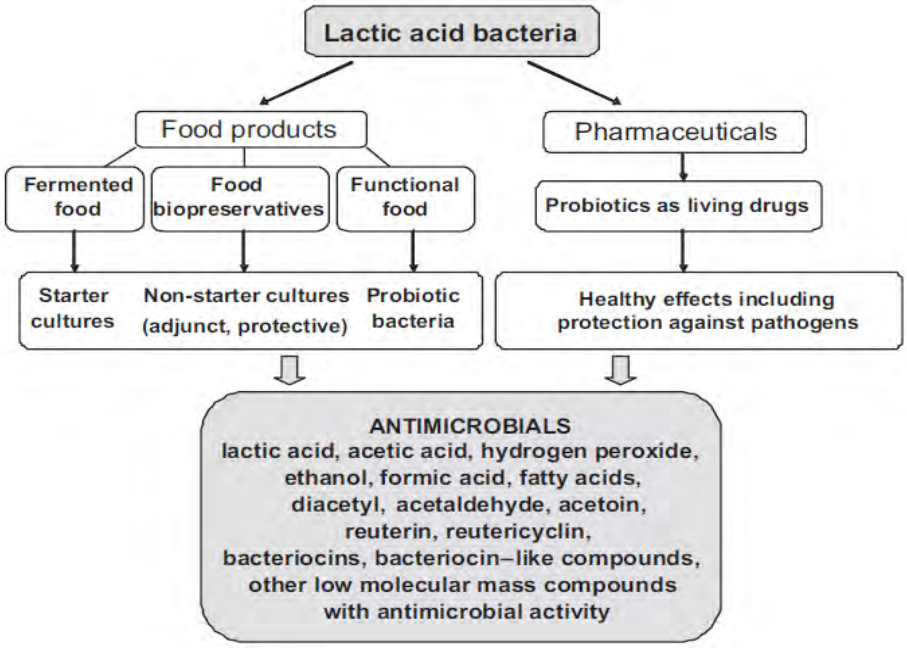


Figure 1.1: The Industrial potential of antimicrobials from the LAB (J.Suskovic et al., 2010).

1.3.1 Antimicrobial protein from Lactic acid bacteria:

Bacteriocins are compounds produced by the bacteria that have a biologically active protein moiety and bactericidal action (Line et al. 2008). Bacteriocins from the LAB are bioactive peptides or proteins with antimicrobial activity toward Gram-positive bacteria, including closely related strains and/or spoilage and pathogenic bacteria. Bacteriocins are ribosomally synthesized and extracellular released bioactive peptides or peptide complexes which have a bactericidal or bacteriostatic effect (Mezaini et al., 2009). There are 2 main reasons for studying bacteriocins in lactobacilli. Firstly, bacteriocin producing starter cultures may result in a more reliable

fermentation process preventing the growth of spoilage bacteria. Secondly, the genetic determinants for bacteriocin production and resistance to bacteriocins have great potential as genetic markers in rDNA technology for application in the future production of food additives or supplements from micro-organisms (Lavanya et al., 2013). The application of antimicrobial-producing lactic acid bacteria or food-grade ferments in the manufacture of industrial food products implies a processing additional advantage to improve the safety and increase the quality of foods, providing an additional way to reduce the likelihood of food-borne diseases (Arques et al., 2015). Antimicrobial substances produced by lactic acid bacteria can be divided into two main groups: low molecular mass substances with molecular mass <1000 Da and high molecular mass substances with molecular mass >1000 Da, such as bacteriocins (J.Suskovic et al., 2010). Again, on the basis of modifications of the precursor peptides, bacteriocins are classified into class I and class II. Class I bacteriocins or lantibiotics undergo posttranslational modifications which introduce the thioether amino acids: lanthionine and methyllanthionine. Class II contains unmodified peptides and is subdivided into four groups: IIa (one-peptide pediocin-like bacteriocin), IIb (two-peptide bacteriocins), IIc (cyclic bacteriocins), and IId (linear in-pediocin-like one-peptide bacteriocins) (Arques et al., 2015). Nisin, produced by *Lactococcus lactis*, is the most thoroughly studied bacteriocin to date and has been applied as an additive to certain foods worldwide (Delves-Broughton et al., 1996).

Table1.1: Classification of bacteriocins from Lactic acid bacteria (Pithva et al., 2011)

I. Lantibiotics	Ribosomally produced peptides that undergo extensive post-translational modification Small (<5 kDa) peptides containing lanthionine and methyl lanthionine Ia. Flexible molecules compared to Ib Ib. Globular peptides with no net charge or net negative charge
II. Nonlantibiotics	Low-molecular-weight (<10 kDa), Heat stable peptides Formed exclusively by unmodified amino acids Ribosomally synthesized as inactive peptides that get activated by post-translational cleavage of the N-terminal leader peptide IIa. Anti-listerial single peptides that contain YGNGGVXC amino acid motif near their N termini IIb. Two peptide bacteriocins IIc. Bacteriocin produced by the cell's general sec-pathway
III. Nonlantibiotics	High-molecular-weight (>30 kDa), heat labile proteins
IV	Complex bacteriocins carrying lipid or carbohydrate moieties, which appear to be required for activity Such bacteriocins are relatively hydrophobic and heat stable

1.3.2 Mode of action of bacteriocins:

Bacteriocins may possess a bactericidal or bacteriostatic mode of action on sensitive cells. Most bacteriocins exert bactericidal mode of action against the sensitive microorganisms, although some have been shown to act in a bacteriostatic manner. The majority of bacteriocins kill susceptible bacteria by membrane permeabilization or by interference with essential enzymes. Nisin forms a complex with ultimate cell wall precursor lipid II, thereby inhibiting the cell wall biosynthesis. Subsequently the complex aggregates and incorporates further peptides to form a pore in the bacterial membrane. Several theories have been proposed to explain the exact mechanism by which antimicrobial peptides kill bacteria. The 'barrel-stave' mechanism describes the formation of transmembrane channels/pores by bundles of peptides. Progressive recruitment of additional peptide monomers leads to steadily increasing the pore size. Leakage of intracellular components through these pores subsequently causes cell death (Pithva et al., 2011).

1.3.3 Effects of antimicrobial peptides on pathogens:

- Antimicrobial effect of nisin V was higher than the one observed with nisin A to control the infection with *L. monocytogenes* in mice (Arques et al., 2015).
- *C. difficile* can take profit from the antibiotic broad spectrum associated disruption of the microbiota and grow and produce toxins in the gut. Lacticin 3147 has the potential to be employed in the treatment of *C. difficile* diarrhea and to eliminate the pathogen when added to an anaerobic fecal fermentation (Arques et al., 2015).
- *Lactobacillus* isolates could protect against *Salmonella typhi* infection through interference with both its growth and its virulence properties, such as adherence, invasion, and cytotoxicity (Daim et al., 2013).
- Aslim et al.investigated the antagonistic effects of *L. bulgaricus* and *S. thermophilus* (production of bacteriocin-like substances) against *H. pylori* and showed that supernatant fluids of these strains had significant antibacterial activity against *H. pylori* (Pithva et al., 2011).
- Antimicrobial activity of probiotic *L. rhamnosus* is associated with cell-free culture filtrate (CFC) and extracellular low molecular weight proteins (EPC) present in CFC filtrate. *L. rhamnosus* produces antimicrobial peptides which inhibit *Escherichia coli, Enterobacter aerogenes, Salmonella typhi, Shigella sp., Proteus vulgaris, Pseudomonas*

aeruginosa, Serratia marcescens, Bacillus cereus, Bacillus megaterium, Bacillus subtilis, and *Staphylococcus aureus* and also reported against *Klebsiella pneumoniae, Helicobacter pylori, Campylobacter jejuni, Micrococcus luteus and Listeria monocytogenes* (Ambalam et al., 2009; Pithva et al., 2011).

1.4 Probiotics:

At the present day, it is important to seek natural bio-preservatives that can control both spoilage and pathogenic microorganisms and keep our food safe. Recently, many studies have revealed that the presence of LAB species with antagonistic activity can improve the quality and safety of meat, dairy products, and other food products (Ibourahema et al., 2008). In the early 20th century, the concept (but not the term) of probiotic was given by Nobel laureate **Élie Metchnikoff**, who postulated that yogurt-consuming Bulgarian peasants lived longer lives (Brown et al., 2006). Probiotics have been defined by The Food Agricultural Organization/World Health Organization (FAO/WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" (Brown et al., 2006). The most commonly consumed probiotics are fermented dairy products such as yogurt and buttermilk. In this aspect, the contribution of lactic acid bacteria to the improvement of food safety and stability of fermented foods has long been known. LAB overgrow other microorganisms of the same ecological niche in food products mainly due to lowering the pH, competing for nutrients, producing various antimicrobial substances such as organic acids, hydrogen peroxide, antibiotics, and bacteriocins. Among these antimicrobials, bacteriocins have received great attention in recent years (Fan & Song, 2013). The term "bacteriocins" was originally coined in 1953 by Jacob to define protein antibiotics of the colicin type, but it is now accepted to include peptide inhibitors from any bacteria (Pithva et al., 2011). To promote host-specific health experiments, the invention of more probiotic strains will be required in the future.

1.4. Lactic acid bacteria as Probiotics:

Probiotic microorganisms are selected based on their long history of use as well as their lack of side effects (Arthure et al., 2002). The probiotic strains must have the indigenous antimicrobial potentiality to work against pathogens. Probiotics have become a major focus of lactic acid bacteria research over the past 10 years, with most attention drawn to the genera *Lactobacillus*

and *Bifidobacterium*. These organisms have been widely reported to exert many beneficial effects, such as activation of the immune system, prevention of cancer cell growth, maintenance of mucosal integrity and presentation of an antagonistic environment for pathogens (Arthure et al., 2002).

Table1.2: Commonly Used Bacterial Strains for Probiotic Purposes (Gismondo, 1999 & Holzapfel, 2001)

Lactobacillus Species	*Bifidobacterium* Species	Other Lactic Acid Bacteria	Non-Lactic Acid Bacteria
L. acidophilus	*B. adolescentis*	*Enterococcus faecium*	*Bacillus subtilis*
L. bulgaricus	*B. animalis*	*Streptococcus thermophilus*	*Escherichia coli strain nisslle*
L. casei	*B. bifidum*		*Saccharomyces boulardii*
L. rhamnosus GG	*B. breve*		*Saccharomyces cerevisiae*
L. plantarum	*B. infantis*		
	B. longum		
	B. thermophilus		

1.4.2 Criteria for an ideal probiotic:

To be beneficial to human health, a probiotic must fulfill several criteria:

- Probiotic bacteria must be of human origin, non-pathogenic and genetically stable (Pithva et al., 2011).
- It must survive passage through the upper gastrointestinal tract (GIT) and must be able to function in the gut environment.
- The strains in probiotic should preferably adhere to the intestinal mucosa so that they are able to colonize the host (Amraii et al., 2014).
- Probiotic bacteria should pass through the highly acidic stomach in order to reach the intestine and create proper conditions for residence (Amraii et al., 2014).
- Probiotics also need to possess the ability to survive and be viable in the products, during food production and storage.
- The probiotic product should be contamination free.

- Therefore, a proper evaluation of each candidate probiotic strain becomes an important step for further implementation as a culture adjunct (Amraii et al., 2014).

1.5 Commercial probiotics:

Live probiotic cultures are generally available in fermented dairy products and probiotic fortified foods. However, there are also tablets, capsules, powders, and sachets available, containing the bacteria in freeze-dried form (Islam et al., 2010). An increasing number of commercial products containing probiotics are available.

1.5.1 Commercial probiotics used in the present study:

Different kinds of products are available that contain probiotic potentialities. In Bangladesh, commercial probiotics are generally available in the local pharmacies. However, these products may not be always easy to find in the market. For this study, only five available probiotics have been selected to test their antimicrobial capability against pathogenic strains and they have been compared with the LAB strains isolated for this study purpose.

1.5.1.1 Prolacto:

Figure 1.2: Prolacto probiotic capsules

Prolacto is probiotic capsules manufactured by Drug International Limited in Gazipur, Bangladesh. In its composition, each capsule contains *Lactobacillus acidophilus* (2 billion), *Bifidobacterium* bifidum (1 billion), *Lactobacillus* bulgaricus (1billion) and fructo-oligosaccharides (100mg).

1.5.1.2 Probio^{TM}:

Probio^{TM} is probiotic capsules manufactured by Square Herbal and Nutraceuticals Limited located in Pabna. Each *Probio*^{TM} capsule contains *Lactobacillus acidophilus* (2 billion), *Lactobacillus bulgaricus* (1billion), *Bifidobacterium bifidum* (1 billion) and Fructo-oligosaccharides (100 mg).

Figure 1.3: Probio™ probiotic capsules

1.5.1.3 TS6 Probiotic Plus:

Figure 1.4: TS6 Probiotic Plus sachet

TS6 Probiotic Plus is manufactured in Taiwan, ROC. It contains 8 probiotic strains (*L. rhamnosus GG, L. paracasei, L. casei, L. acidophilus, Lactococcus latis, B. bifidum, B. longum, B. infantis*). Its composition also holds traces of Perilla leaf extract, Pine bark extract and Oligosaccharide.

1.5.1.4. Prokids Probiotic:

Figure 1.5: Prokids probiotic sachet

Prokids Probiotic is manufactured by Fu-E Life sciences Co., Ltd. Taiwan. Its composition includes 9 lactic acid bacterial strains: *L. casei, L. acidophilus, L. rhamnosus, Lactococcus latis, L. helveticus, L. fermentum, L. paracasei, L. plantarum, Bifidobacterium bifidum.*

1.5.1.5 Emmi Swiss Premium Yogurt: Emmi Swiss Premium Yogurt is a low-fat yogurt with 1.4% fat and made from Swiss Milk, with 10% Aloe Vera extract. It is produced by Emmi Schweiz AG, 6002 Lucerne, Switzerland.

Figure: 1.6: Swiss premium yogurt Aloe Vera

Main ingredients of this probiotic are fresh Swiss milk, sugar, milk proteins, live lactic cultures (*Sc. thermophilus, Lb. bulgaricus, Lb. acidophilus*), and fruit preparations.

Studies showed that commercial probiotic consumption often increases specific intestinal microflora, but usually not the total count of bacteria found in the intestine. It is also evident in the majority of reported research cases that specific bacteria do not increase unless subjects consume very high dosages of probiotics in the form of supplements, not those naturally found in foods (Brown et al., 2006). This study will discuss some of these commercial probiotics available in Bangladesh along with their antimicrobial potentiality to provide health benefits to consumers.

1.6 Objective of the study:

In a country like Bangladesh, the habit of using probiotics as a dietary supplement is less common. The reason behind this may include a less availability of native quality products and a relatively higher price for the available foreign supplied probiotics. However, the practice of having probiotics in Bangladesh can be ensured if new probiotics with the same properties, at a lower price can be industrially manufactured. Therefore, the objective of this study involves partially validating the previous project named "Screening of Lactic Acid Bacteria with Antimicrobial Property Isolated from Fermented Foods" to find out antimicrobial properties present in Lactic acid bacteria strains isolated from fermented vegetables and dairy products. Finally, using the isolated LAB strains an economically affordable and low-cost Probiotic Yogurt Starter Culture will be prepared.

Chapter 2:
Materials and methods

2. Materials and methods

2.1 Working place for the study:

The present research work was performed in the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka 1212, Bangladesh.

2.2.1 Collection and processing of Lactic acid bacteria isolates:

As mentioned before, Lactic acid bacteria are commonly present in fermented beverages and dairy products, meat, fruits, vegetables, etc. These are the sources of the LAB outside the host's body. Among these, the LAB has been isolated from fermented vegetables and dairy products which were obtained randomly from local markets of Bangladesh.

Table 2.1: Sources of LAB isolates

Sl. No.	Sources	Types of sample
1.	MatriVandar Sweet Curd	Yogurt
2.	Aarong Dairy Sour Curd (Light yellowish colony)	Yogurt
3.	Aarong Dairy Sour Curd (white colony)	Yogurt
4.	Shakti Sweet (white colony)	Yogurt
5.	Shakti Sweet (white colony)	Yogurt
6.	Delicia sweet curd (white colony)	Yogurt
7.	Buttermilk (white colony)	Dairy product
8.	Buttermilk (off-white colony)	Dairy product
9.	Dhaka Cheese	Dairy product
10	Cauliflower (white colony)	Vegetable
11.	Cauliflower (light yellowish colony)	Vegetable
12.	Turnip (white colony)	Vegetable
13.	Turnip (light yellowish colony)	Vegetable
14.	Carrot (white colony)	Vegetable
15.	Carrot (light yellowish colony)	Vegetable
16.	Brinjal (off-white colony)	Vegetable
17.	Brinjal (white colony)	Vegetable
18.	Cabbage (off-white colony)	Vegetable
19.	Cabbage (light yellowish colony)	Vegetable
20.	Cabbage (white colony)	Vegetable

As this has to be a running project, the LAB isolates have been obtained from its stored pure stocks from the previous study. The previous study involved isolation of LAB from 20 samples. These 20 isolates from 20 samples were cultured and stored inside vials in T1N1 media with paraffin oil for further uses.

The above-mentioned samples were processed for obtaining LAB isolates. In the case of solid samples, fermentation was done by cleaning and dicing the samples and then they are placed in distilled water. The samples with distilled water were kept at room temperature. After three to four weeks, 1ml of this fermented samples were directly inoculated into MRS (de Man, Rogosa, Sharpe) broth and incubated for 48 hours at 30 °C. Then, 1 ml of this MRS broth was placed into MRS agar plate using a pipette and incubated for 48 hours at 30 °C. Firstly a serial dilution of x2 was prepared using 0.9% saline solution. Two hundred microliter of this was dropped onto an MRS agar plate, and then the spread plate technique was applied. Distinct colonies were found after 48-72 hours of anaerobic incubation at 30 °C. The cultured LAB strains were used several times for the previous project and then stored inside small vials in T1N1 media with paraffin oil at room temperature.

2.2.2 Preparing LAB isolates for the present study:

The present study started with doing subculture of the LAB from the stored stock of the LAB isolates.

- All the stored samples in vials kept at room temperature were examined before subculture.
- MRS (de Man, Rogosa, Sharpe) agar media were prepared.
- A small amount of inoculum was taken from a vial with a sterile inoculating loop and streaked on a labeled MRS agar plate.
- The inoculated plates were incubated at 37 ºC for 48 hours (Yang et al., 2012).
- In this way, all the 20 stocks with isolates were grown on MRS agar media.
- Visible distinct colonies were observed after every subculture on most of the plates.
- All isolates used in the present study were kept active and preserved through subculture after every 2-3 weeks prior to performing the experiments.

However, all the 20 isolates did not appear with satisfied colonies after incubation. Different parameters were also used for maximum growth of these isolates. The **Table 2.2** below shows 10 such isolates that gave satisfactory growth on MRS plates. Rest of the plates after inoculating from the stocks and incubation did not show distinct colonies and therefore, were excluded from this study.

Table 2.2: Final isolates after subculture

Sl. No.	Sources	Initials	Types of sample
1.	Matri Vandar Sweet Curd (white colony)	M	Yogurt
2.	Aarong Dairy Sour curd (Light yellowish colony)	A.L	Yogurt
3.	Dhaka Cheese (white colony)	D.Ch	Dairy product
4.	Brinjal (white colony)	Br.W	Vegetable
5.	Brinjal (off-white colony)	Br.OW	Vegetable
6.	Cauliflower (white colony)	Cli.W	Vegetable
7.	Cauliflower (light yellowish colony)	Cli.L	Vegetable
8.	Turnip (white colony)	T	Vegetable
9.	Cabbage (light yellowish colony)	Cg.L	Vegetable
10.	Carrot (white colony)	Ca.W	Vegetable

2.3 Testing antimicrobial properties:

2.3.1 Agar well diffusion bioassay:

An agar well diffusion assay (AWDA) was first described by Nathan et al. in 1978. In this study, the agar well diffusion method has been used to detect the antimicrobial activities of the cell-free supernatants (CFS) produced by lactic acid bacterial strains isolated from vegetables and dairy products. The experiment was performed under strict aseptic conditions.

- The 10 final LAB isolates were freshly sub-cultured. A small loop of colonies was taken from each of the isolates and inoculated into falcon tubes containing 10 ml of freshly made and autoclaved MRS broth.

- The 10 LAB inoculated MRS broth tubes were incubated in a shaker incubator at 37 ºC for 48 hours.
- After 48 hours, each LAB isolates were grown in MRS broth and each of the culture in falcon tubes was centrifuged for 15 minutes to get the cell-free supernatant (CFS).
- Muller Hinton Agar (MHA) was poured into every petri dish and labeled for separate pathogenic organisms. Each plate contained about 30 ml of MHA media.
- Ten indicator organisms were selected: *Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus pneumoniae, Bacillus subtilis, Bacillus cereus, Shigella dysenteriae, Shigella flexneri, Klebsiella sp., Enterobacter cloacae,* and *Salmonella typhi.*
- The selected indicator organisms were 24-hour pure culture so that they provide active strains suitable for using in agar well diffusion method.
- A small loop of each indicator organism was dipped in 0.9% saline and vortexed for mixing.
- A sterile Q-tips cotton swab was dipped into the saline containing an indicator organism.
- The wet cotton swab was rubbed properly on the surface of the MHA plate and the tips were immediately disposed of into disinfectant.
- All the MHA plates were well-dried.
- After the medium was dried, wells of 7 mm in diameter were cut in the MHA plates with a sterile borer.
- Into each well 50 µl of the cell-free supernatants were placed. Every plate had 4-5 wells with LAB supernatants.
- All the MHA plates were incubated at 37 ºC for 24 hours for desirable results.
- Microbial growth was determined by measuring the diameter of the zone of inhibition.

2.3.2 Measuring pH:

Fermented foods are less perishable than the original raw materials. Their nutritional value may be enhanced and the safety of these foods may be improved due to the inhibition of pathogenic bacteria by the low pH and the presence of organic acids and antimicrobial compounds. The ability of lactic acid bacteria to regulate their cytoplasmic or intracellular pH is one of the most

important physiological requirements of the cells (Hutkins and Nannen, 1993). In this study, the search of antimicrobial property includes measuring the pH of the cell-free supernatant of the lactic acid bacteria.

Measuring pH from Cell-Free Supernatant:

- Ten LAB isolates were grown in MRS broth. Each isolate was inoculated in 100 ml of MRS broth in a conical flask.
- The conical flasks with the broth were incubated in a shaker incubator at 37 ºC for 48 hours.
- After incubation, every broth cultures were taken in 10 ml falcon tube and centrifuged for 15 minutes at 10,000 rpm.
- Cell-free supernatants were obtained after centrifugation. The supernatant of each isolate was poured into a beaker.
- The pH meter was calibrated before use.
- Fresh, unused, unexpired pH buffers must be used for calibration. Buffers should be at the same temperature as the testing solutions.
- The pH electrode was rinsed with distilled water and then with the buffer being used for calibration (*i.e.*, pH 7.00).
- The pH electrode was dipped into a neutral pH buffer (*i.e.*, pH 7.00). The buffer was stirred with a magnetic bar for best results.
- The "CAL/MEAS" button was pressed to select the 'calibration (standardization)' function. The buffer pH value was set on the meter to display 7.00.
- The pH electrode was rinsed with distilled water and then dipped into each MRS broth culture and stirred well for results.
- The value displayed on the pH meter was noted down and pH of the rest of the solutions was measured in the same way.
- The pH electrode needs to be thoroughly rinsed between measurements with distilled water to prevent possible contamination of the tested solutions.
- The electrode was gently blotted on a laboratory cleaning tissue to remove the excess rinse water.
- One should not rub the bulb since this can cause a static charge buildup.

- pH of all the CFS of 10 isolates was measured and noted down.

2.4 Comparison with commercial products:

2.4.1 Agar well diffusion:

The concept of probiotics describes that some substances secreted by one microorganism can stimulate the growth of another microorganism. These substances are the antimicrobial compounds that are able to inhibit the growth of other microbes or kill them. In the present study, the antimicrobial properties of such strains have been monitored through the agar well diffusion method. This section will screen for the antimicrobial compounds secreted from LAB strains derived from some available commercial probiotics**.** Five previously explained commercial probiotics have been used for this purpose.

- For checking the antimicrobial potentiality of commercial probiotics, the strains present in these products must be given to growing in a media, which is MRS broth, suitable for lactic acid bacteria strains.
- Five commercial probiotics were: Prokids Probiotic, Prolacto, Probio, TS6 Probiotic Plus and Emmi Swiss Premium Yogurt.
- Every probiotic were measured 0.25 gm for inoculation.
- In a conical flask, 50 ml of MRS broth was taken and 0.25 gm of a probiotic was inoculated.
- In this way, 5 conical flasks were prepared to contain 0.25 gm of probiotic powder of each type.
- All the conical flasks with MRS broth were incubated in a shaker incubator at 37 ºC for 48 hours.
- After 48 hours, all the probiotics were grown in MRS broth and each probiotic culture was taken in falcon tube for centrifugation (at 10,000 rpm, for 15 minutes) to get the cell-free supernatant (CFS).
- For doing agar well diffusion with commercial probiotics against indicator organisms, 5 pathogenic strains were selected: *Salmonella typhi, Shigella dysenteriae, Shigella flexneri, Bacillus subtilis* and *Klebsiella pneumoniae.*

- Twenty-four-hour pure culture of these selected indicator organisms were taken for the test to get active strains suitable for the experiment.
- A small loop of each indicator organism was dipped in 0.9% saline and vortexed for mixing.
- A sterile cotton swab was dipped into the saline containing an indicator organism.
- The wet cotton swab was rubbed properly on the surface of the MHA plate and the tips were immediately disposed of into disinfectant.
- All the MHA plates were well-dried.
- After the medium was dried, wells of 7 mm in diameter were cut in the MHA plates with a sterile borer.
- Each well-contained 50 µl of the probiotic cell-free supernatants. Every plate had 5 wells with 5 different probiotic supernatants.
- All the MHA plates were incubated at 37 ºC for 24 hours for desirable results.

2.4.2 Cross Streak Method (CSM):

There are many techniques for detecting antimicrobial activity and most of them are based on methods involving diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms (Pereira and Kamat, 2011). The cross-streaking is an easy and relatively rapid method for identifying inhibitory properties of any sample against any bacterium or mold which will grow discretely on an agar plate.

- The same 5 indicator organisms were selected for this cross streak method.
- The antimicrobial activity of the 5 commercial probiotics was supposed to be screened against these 5 pathogenic strains in this experiment.
- Some markings were drawn on a transparent sheet which acted as a template and was attached below the plate to facilitate streaking and thereafter in quantifying the results.
- The markings were 5 straight lines, with a 1cm gap between each other on a round plate.
- The 5 straight lines denote P_1, P_2, P_3, P_4 and P_5 for pathogenic strains.
- Again, perpendicular lines were drawn over previous 5 lines denoting samples S_1, S_2, S_3, S_4 and S_5 for the 5 probiotics.
- The 5 commercial probiotics grown on MRS broth were directly used in this experiment.

- In a 30 ml Muller Hinton Agar (MHA) plate, streaking was done with a sterile loop over the template drawn under the MHA plate.
- The MHA plate was incubated at 37 ºC for 24 hours.

2.5 Preparation of the Probiotic Yogurt Starter Culture:

Lactic acid bacteria (LAB) species have become an industrially important group of bacteria with multiple benefits. If these strains, mostly isolated from vegetables, are used for manufacturing commercial probiotics as fermented dairy products, they would be able to provide health benefits to the general mass of the country. The preparation of probiotic yogurt in the process described below involves inoculation of LAB isolates derived from vegetables and some milk products.

2.5.1 Inoculum preparation (single isolate):

- Three types of milk were chosen for preparing inoculum: Whole milk, powdered milk and skimmed milk.
- Six small beakers were cleaned with distilled water. Two beakers were taken for each type of milk and labeled with ‘Sugar’ and ‘No sugar’.
- In two beakers, 20 ml of whole milk were poured and in the beaker labeled as ‘Sugar’, 0.93 gm of sugar was added.
- The sugar was stirred to mix with the milk well.
- The same procedure was followed for the two other types of milk. That is, 20 ml of powdered milk were taken in two separate beakers and 0.93 gm sugar was added to one of them. Again, 20 ml of skimmed milk were taken in two separate beakers and the same amount of sugar was added to one of the beakers.
- In total, six small beakers were prepared and three of them contained sugar.
- From the 10 LAB isolates, a single vegetable derived strain has been selected to inoculate the milk samples. Strains from Brinjal (white colony) were chosen at first for single inoculation.

- Under the aseptic condition, single white colonies of Brinjal were taken with a sterilized inoculating loop and inoculated into all the 6 beakers.
- These six inoculated beakers were incubated at 42 ºC for 4-5 hours.
- After incubation, inoculums were ready to inoculate for yogurt preparations.

(a) (b) (c)

Fig. 2.1: Inoculum preparation. (a) Inoculation of single LAB isolates into 20 ml of whole milk. (b) Inoculation of single LAB isolates into 20 ml of powdered milk. (c) Inoculation of single LAB isolates into 20 ml skimmed milk.

2.5.2 Inoculating milk with prepared inoculums:

- For developing yogurt, six medium sized beakers were required. These beakers were filled with 200 ml of milk. Two of them contained whole milk and with sugar in one of them. Two of them contained powdered milk with sugar in one of them and the remaining two beakers contained skimmed milk with sugar in one of them.
- Here, amount of added sugar in 3 types of milk was 9.3 gm (For 20 ml of milk, 0.93 gm sugar. Therefore, for 200 ml of milk, 9.3 gm sugar).
- The beakers were labeled according to the mixture present in it.
- The six freshly made inoculums were taken out from the incubator and poured into the respective beakers containing 200 ml milk.
- All the milk samples were stirred well with a sterilized glass rod.
- These inoculated milk samples were kept at 42 ºC for 4 hours to overnight incubation.
- The milk samples were also monitored after a regular interval.
- Overnight incubation would develop yogurt formation in milk samples.

- After incubation, all the six beakers were taken out from the incubator for observation.

2.5.3 Dehydration of produced yogurt:

- The six samples derived from the previous procedure were found to produce yogurt after incubation.
- From each of the six newly prepared yogurt samples in the beakers, approximately 2 gm of yogurt were taken for dehydration.
- This dehydrating step involves, spreading 2 gm of yogurt on a clean aluminum foil paper a very thin layer that can ensure powder formation after drying.

Fig. 2.2: Spreading 2 gm of yogurt on clean aluminum foil paper before dehydration

- After spreading on the foil papers labeling was done for six types of milk samples following initial methods
- All the six foil papers were kept at 65 ºC for 2-3 hours.
- The sample on foil papers was continuously monitored after a regular interval.
- The foils were taken out of the incubator after drying and kept in normal temperature for cooling.

2.5.4 Extraction of yogurt powder:

- Incubation at 65 ºC caused the thin layers of yogurt to become dry on the foil paper.
- On a cleaned bench top, the foil papers were kept.
- A sterile spatula was rubbed over the foil paper very carefully to extract the yogurt.
- Gradually, the yogurt came out like fine powder from the foil paper.

- Yogurt samples that generated yogurt powder were stored in sterilized microcentrifuge tubes.

2.5.5 Inoculating yogurt powder into milk:

- In this step, the potentiality of the yogurt powder has been checked whether it can form yogurt again.
- Two kinds of milk were taken for this purpose, i.e. 50 ml of whole milk and 50ml of powdered milk.
- In one beaker, 2.32 gm sugar was added with whole milk and labeled. Another beaker contained 50 ml of whole milk which was without sugar.
- In another beaker, 2.32 gm sugar was added with powdered milk and another beaker contained 50 ml of powdered milk which was without sugar.
- From sugarless whole milk and powdered milk, 0.25 gm of yogurt powder was derived and they were measured for inoculation.
- Again, 0.25 gm of yogurt powder derived from sugar added whole milk and powdered milk were measured for inoculation.
- In 50 ml of sugarless whole milk, 0.25 gm sugarless whole milk yogurt powder was inoculated and in 50 ml of sugarless powdered milk, 0.25 gm sugarless powdered milk yogurt powder was inoculated.
- Again, in 50 ml of sugar added whole milk, 0.25 gm sugar added whole milk yogurt powder was inoculated and in 50 ml of sugar added powdered milk, 0.25 gm sugar added powdered milk yogurt powder was inoculated.
- These four beakers were kept at 42 ºC for 4 hours to overnight incubation.

2.5.6 Preparing yogurt and yogurt powder with mix cultures:

- Fifty milliliters of whole milk was taken without sugar and labeled.
- Freshly sub-cultured 10 LAB isolates of this study were prepared.
- Under the aseptic condition, 1 loop (10 µl approx.) of bacteria were taken from single colonies with a sterile inoculating loop and inoculated into 50 ml of whole milk.
- In the same way, the rest of the isolates were also inoculated into the whole milk.

- The beaker was incubated at 42 ºC for 4 hours to overnight incubation.

Fig. 2.3: 20 ml of whole milk inoculated with 10 LAB isolates

- The milk sample was found to produce yogurt after incubation.

- Extraction of yogurt powder from mix cultures was done by spreading a very thin layer of 2 gm of the newly formed yogurt on a clean foil paper.
- The foil paper was kept at 65 ºC for 2-3 hours.
- The sample on foil paper was continuously monitored after a regular interval.
- The foil paper was taken out of the incubator after drying and kept in normal temperature for cooling.
- Incubation at 65 ºC caused the thin layers of yogurt to become dry on the foil paper.
- A sterile spatula was rubbed over the foil paper very carefully to extract the yogurt powder.
- Slowly, the yogurt came out like fine powder from the foil paper.
- Yogurt samples that generated yogurt powder were stored in a sterilized microcentrifuge tube and labeled.
- This extracted powder was again inoculated into 50 ml of whole milk.
- It was seen that, this powder derived from mix cultured yogurt was also able to produce yogurt when inoculated in whole milk.

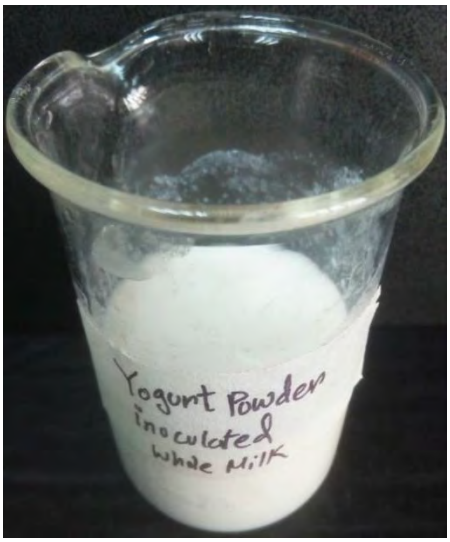


Fig. 2.4: 50 ml whole milk inoculated with mix cultured yogurt powder

2.6 Yogurt formation using commercial probiotics:

- In this method, the lactic acid bacterial strains present in the 5 commercial probiotics were given to grow in milk samples.
- For this, 50 ml of milk were taken in beakers for each probiotic.
- Like previous methods, 0.25 gm of every probiotic was inoculated into 50 ml of milk.
- The 5 probiotic inoculated milk samples were kept at 42 ºC for 4 hours to overnight incubation. After incubation, beakers were taken out for observation.

2.7 Strain preservation:

The isolates were stored in T1N1 vials. The T1N1 was prepared by adding 1 gm of Tryptone casein digest, 1 gm of NaCl and 0.6 gm of agar powder. The mixture was boiled and poured into small glass vials. Later, the vials were autoclaved and allowed to solidify. After it solidified, the bacterial inoculum was taken using a sterile needle and stabbed on the media. It was kept in the incubator for overnight incubation. On the next day, 400 µl of sterile paraffin oil was added on top of the agar. The vials were tightly capped and stored at room temperature. In this way the isolates can be preserved for many days for further research.

Chapter 3:

Results

3. Results

3.1 Growth of Lactic acid bacteria isolates for the present study:

Lactic acid bacteria were isolated from vegetables and dairy products in the previous project. The project isolated 20 LAB isolates from different sources. After regular sub-culture from the 20 stocks, 10 LAB isolates have been found to have maximum growth on MRS agar plates after incubation. Only these 10 isolates were used in further experiments.

For the maximum growth of lactic acid bacteria, different parameters were used. Initially, the MRS plates were incubated at 40 ºC for 24 hours. Again, 30 ºC temperatures for 24 hours were also given to monitoring for some isolates. During the end of the project work, sub-cultures were incubated at 37 ºC for 48 hours. Finally, this temperature suited maximum isolates and 10 such LAB isolates were selected.

Table 3.1: 10 LAB isolates after sub-culture

Sl. No.	Sources
1.	Matri Vandar Sweet Curd (white colony)
2.	Aarong Dairy Sour curd (Light yellowish colony)
3.	Dhaka Cheese (white colony)
4.	Brinjal (white colony)
5.	Brinjal (off-white colony)
6.	Cauliflower (white colony)
7.	Cauliflower (light yellowish colony)
8.	Turnip (white colony)
9.	Cabbage (light yellowish colony)
10.	Carrot (white colony)

Figure 3.1(a, b, c & d) shows growth of lactic acid bacteria of some isolates on MRS agar plates. Most of the strains have growth with visible white, off-white or light yellowish colonies.

Fig. 3.1(a): LAB isolated from Matri Vandar Sweet Curd on MRS media

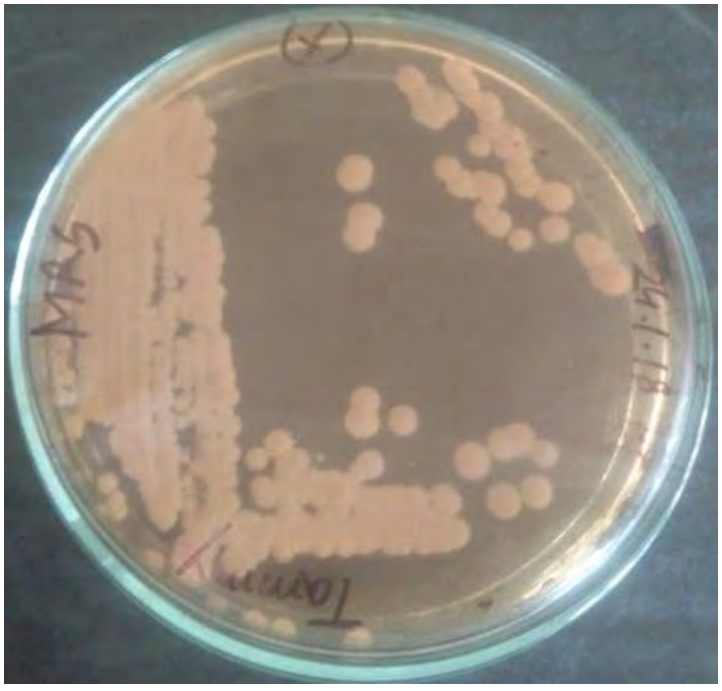


Fig. 3.1(b): LAB isolated from Cauliflower showing light yellowish colonies on MRS media

Fig. 3.1(c): LAB isolated from Aarong Dairy Sour curd shows light colonies on MRS media

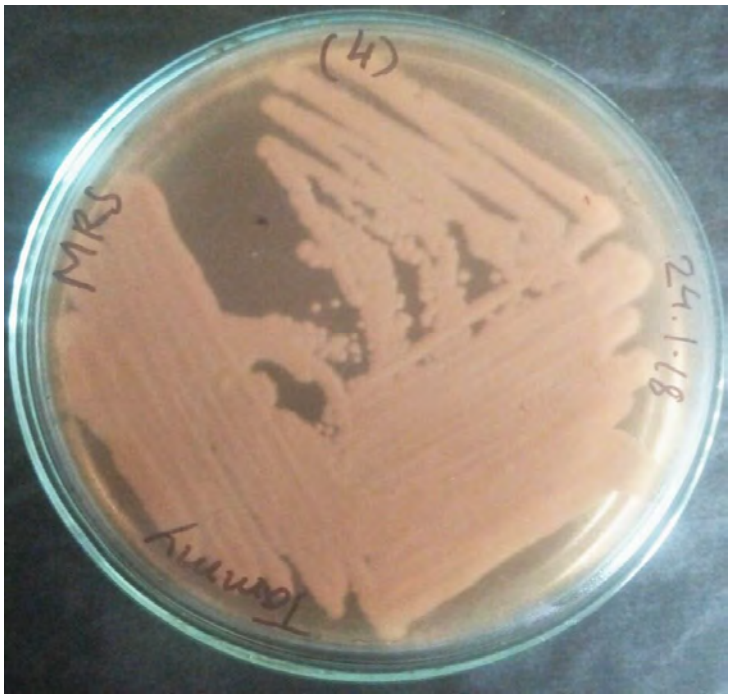


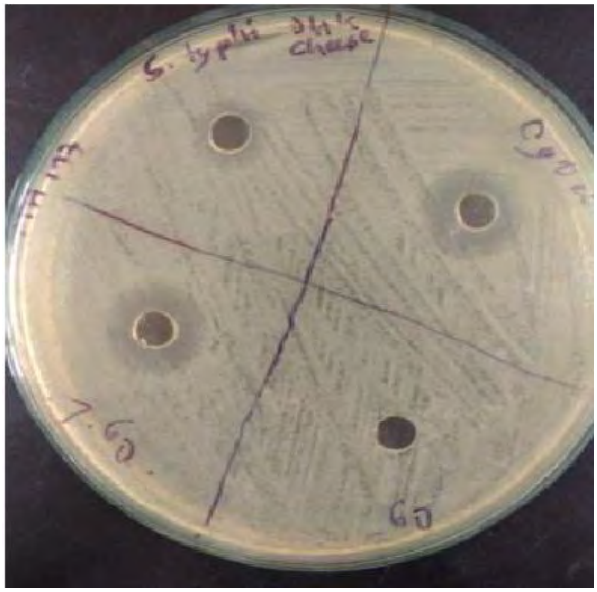
Fig. 3.1(d): LAB isolated from Brinjal showing white colonies on MRS media

3.2 Determination of antimicrobial properties of the LAB:

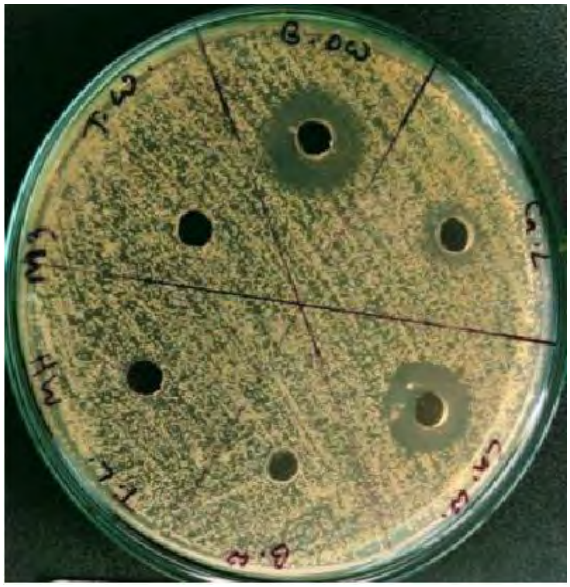
Antimicrobial properties of the LAB were identified through Agar Well Diffusion method and by calculating the pH of the 10 LAB isolates. The result signifies the activity of lactic acid bacteria against some selected indicator organisms.

3.2.1 Results on Agar Well Diffusion Bioassay:

Agar well diffusion method measures antimicrobial activity that involves the formation of Zone of inhibition (ZOI). Zone of inhibition (ZOI) observed from different samples on the MHA plates using agar well diffusion method against ten pathogenic organisms. Some of the results are shown by the figure below.



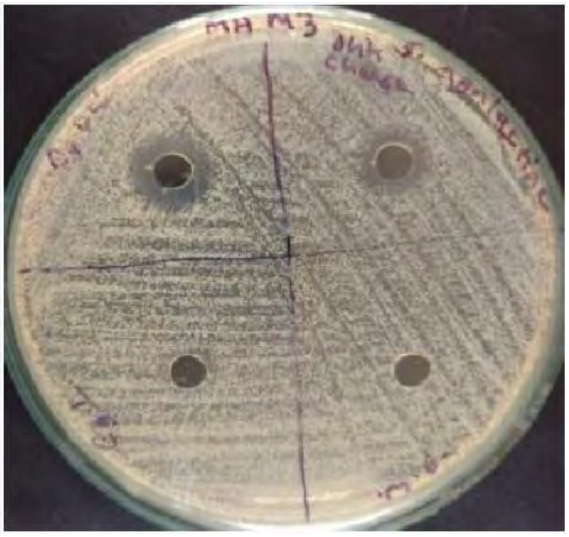
Four isolates against *Salmonella typhi*



Six isolates against *Streptococcus pneumoniae*

Four isolates against *Enterobacter cloacae*

Four isolates against *Streptococcus pyogenes*



Four isolates against *Streptococcus agalactiae*

Four isolates against *Klebsiella sp*

Fig. 3.2: Zone of inhibition by the LAB isolates against indicator organisms

Table 3.2: Antimicrobial activity of Cell-free supernatant from 10 LAB isolates against tested organisms:

Name of the tested organism / Sources of LAB isolates		Zone of inhibition (ZOI) by indicator organisms (millimeter)									
		Salmonella typhi	***Enterobacter cloacae***	***Shigella flexneri***	***Shigella dysenteriae***	***Klebsiella sp.***	***Streptococcus agalactiae***	***Streptococcus pneumoniae***	***Streptococcus pyogenes***	***Bacillus cereus***	***Bacillus subtilis***
1.	**Matri Vandar Sweet Curd (white colony)**	No zone	No zone	No zone	No zone	No zone	No zone	No zone	12	11	11
2.	**Aarong Dairy Sour Curd (Light yellowish colony)**	15	11	No zone	10	11	No zone	No zone	13	No zone	No zone
3.	**Dhaka Cheese (white colony)**	10	15	10	No zone	12	12	No zone	13	No zone	15
4.	**Brinjal (white colony)**	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone
5.	**Brinjal (off-white colony)**	No zone	No zone	No Zone	No zone	10	No zone	16	No zone	No zone	11
6.	**Cauliflower (white colony)**	10	13	14	14	11	No zone	14	14	15	14
7.	**Cauliflower (light yellowish colony)**	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone
8.	**Turnip (white colony)**	No zone	10	No zone	No zone	No zone	No zone	No zone	No zone	No zone	No zone
9.	**Cabbage (light yellowish colony)**	18	15	12	10	14	No zone	No zone	14	No zone	18
10	**Carrot (white colony)**	No zone	13	No zone	No zone	10	No zone	15	No zone	No zone	10

3.2.2 pH of lactic acid bacteria isolates:

Lactic acid bacteria produce acid during fermentation that causes a lower pH of the growth environment. pH of LAB isolates was measured with a pH meter, resulted in different pH for cell-free supernatant of LAB isolates. **Table 3.3** shows the respective pH values of 10 LAB isolates.

Table 3.3: pH of cell-free supernatant from LAB isolates

Sl. No.	LAB Sources	Type of sources	pH of LAB cell-free supernatant
1.	Matri Vandar Sweet Curd	Yogurt	5.9
2.	Aarong Dairy Sour curd (Light yellowish colony)	Yogurt	4.6
3.	Dhaka Cheese (white colony)	Dairy product	4.9
4.	Brinjal (white colony)	Vegetable	5.4
5.	Brinjal (off-white colony)	Vegetable	4.9
6.	Cauliflower (light yellowish colony)	Vegetable	5.8
7.	Cauliflower (white colony)	Vegetable	5.4
8.	Turnip (white colony)	Vegetable	4.9
9.	Cabbage (light yellowish colony)	Vegetable	4.9
10.	Carrot (white colony)	Vegetable	4.6

3.3 Examining antimicrobial potentiality of commercial probiotics:

3.3.1 Agar well diffusion: Probiotics contain lactic acid bacteria in their formulations. These strains must be active to show antimicrobial activity in the human gut. Agar well diffusion was done with CFS produced by 5 commercially available probiotics against some tested organisms. The results are shown in the figures below.

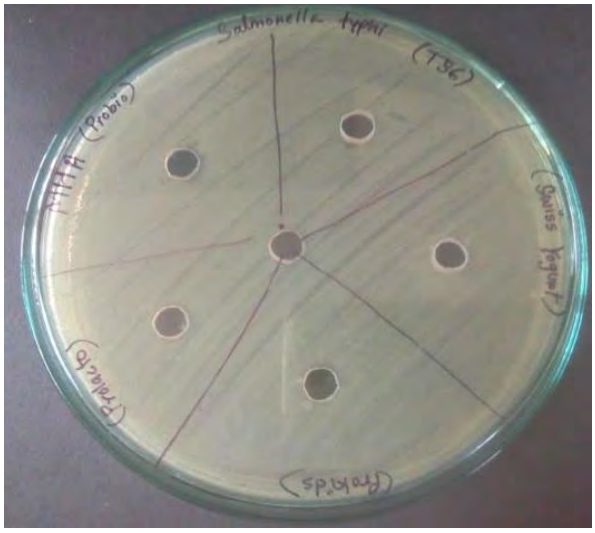


Fig.3.3 (a): CFS of 5 probiotics has no ZOI against *Salmonella typhi*

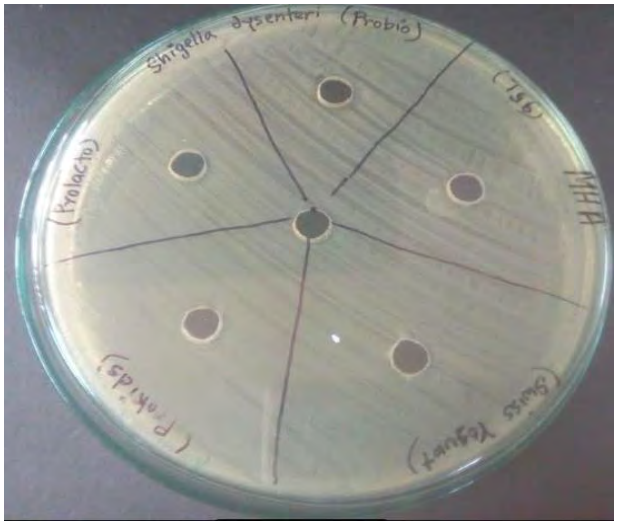
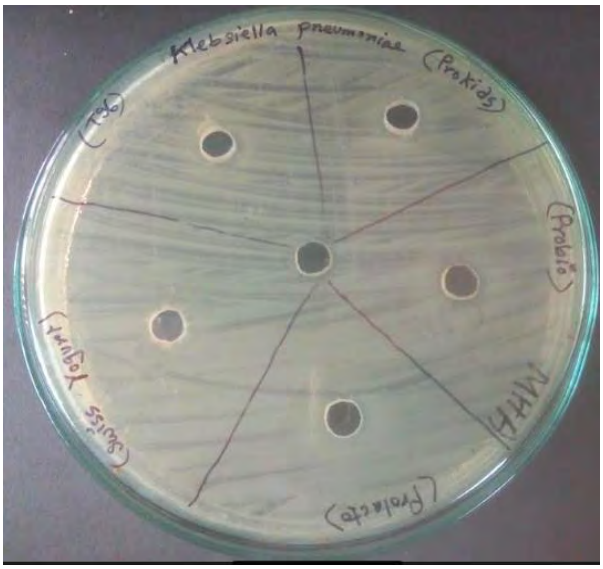


Fig.3.3 (b): CFS of 5 probiotics has no ZOI against *Shigella dysenteriae*



(c) (d)

Fig.3.3: (c) CFS of 5 probiotics has no ZOI against *Klebsiella pneumoniae*

(d) CFS of 5 probiotics has no ZOI against *Shigella flexneri*

Fig.3.3 (e): CFS of 5 probiotics has no ZOI against *Bacillus subtilis*

3.3.2 Results of Cross streaking:

The antimicrobial activity of the 5 commercial probiotics was examined against the same 5 pathogenic strains: *Salmonella typhi, Shigella dysenteriae, Shigella flexneri, Bacillus subtilis, Klebsiella pneumonia*. The figure below shows the result of cross streaking.

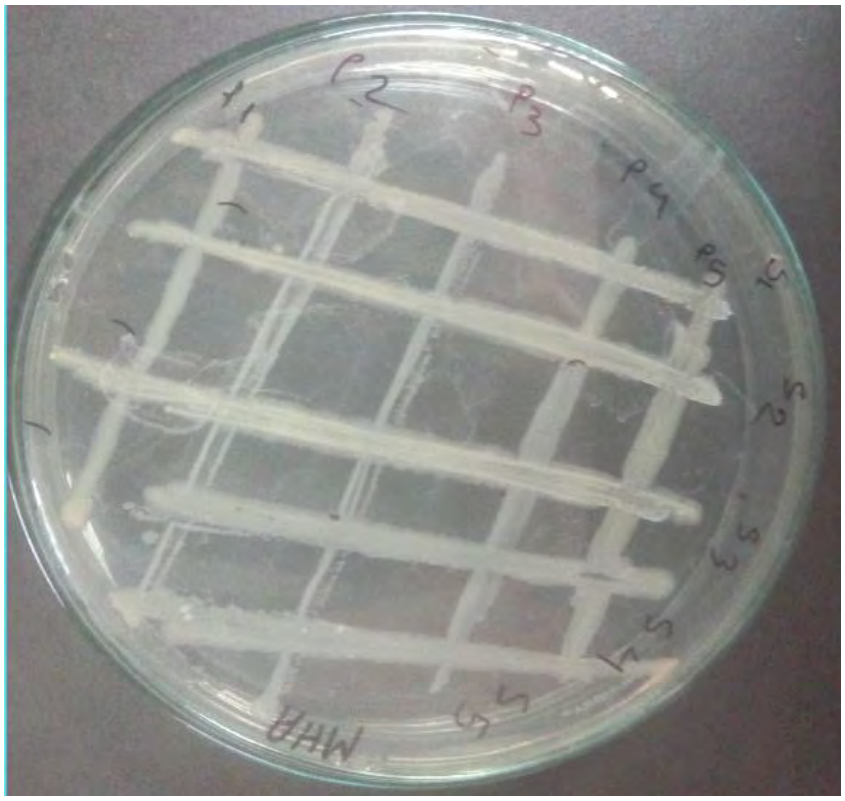


Fig. 3.4: Cross streaking done with 5 probiotics (grown in MRS broth) against 5 indicator organisms showed no inhibition area

3.4 Probiotic Yogurt Formulations:

3.4.1 Single isolate yogurt:

For preparing single isolate yogurt, bacteria from Brinjal (white colony) were inoculated into milk to make the inoculums or starter culture. 20 ml inoculums with three types of milk were inoculated into 200 ml of milk samples for yogurt production. Three types of milk involved whole milk, powdered milk and skimmed milk having sugar in one of their samples. After incubation, all the six beakers were found to produce a good quality yogurt. Figures of all the yogurts are given below.

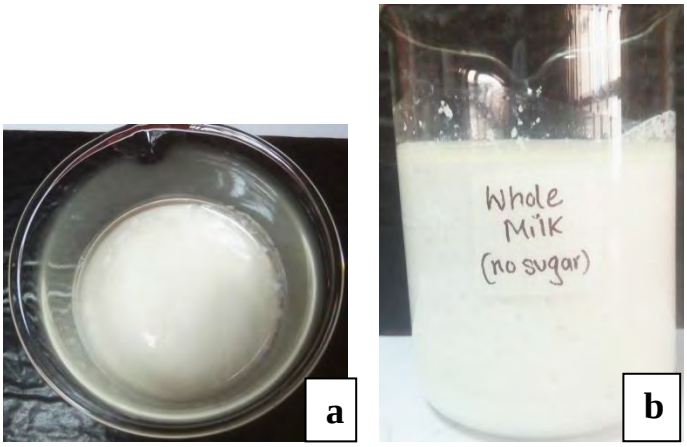


Fig.3.5: (a) Yogurt formation with single isolate in whole milk (top view), (b) Side view

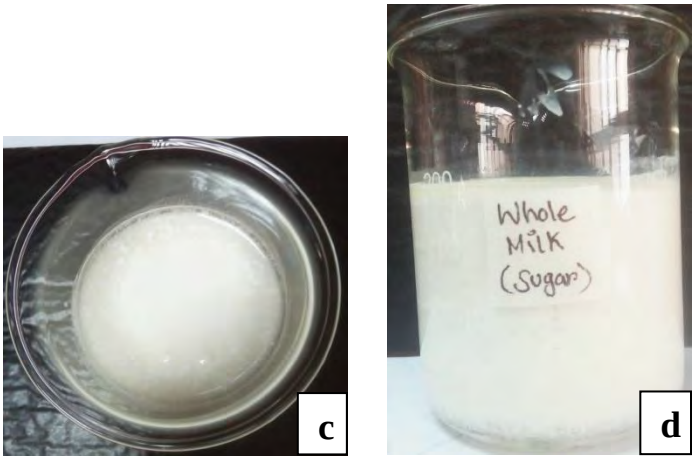


Fig.3.6: (c) Yogurt formation with single isolate in sugar added whole milk (top view),

(d) Side view

Fig.3.7: (e) Yogurt formation with single isolate in powdered milk (top view), (f) Side view

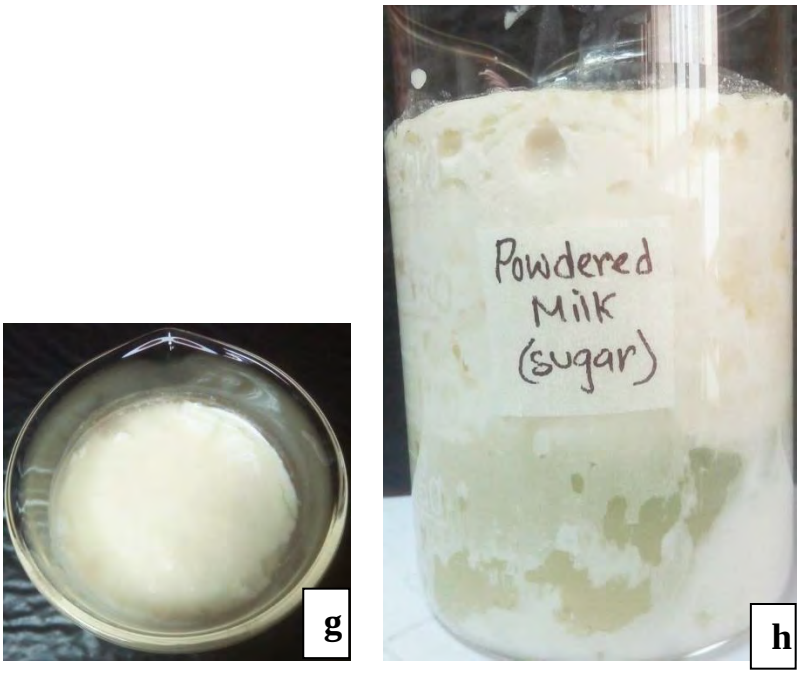


Fig.3.8: (g) Yogurt formation with single isolate in sugar added powdered milk (top view),

(h) Side view

Fig.3.9: (i) Yogurt formation with single isolate in skimmed milk (top view), (j) Side view

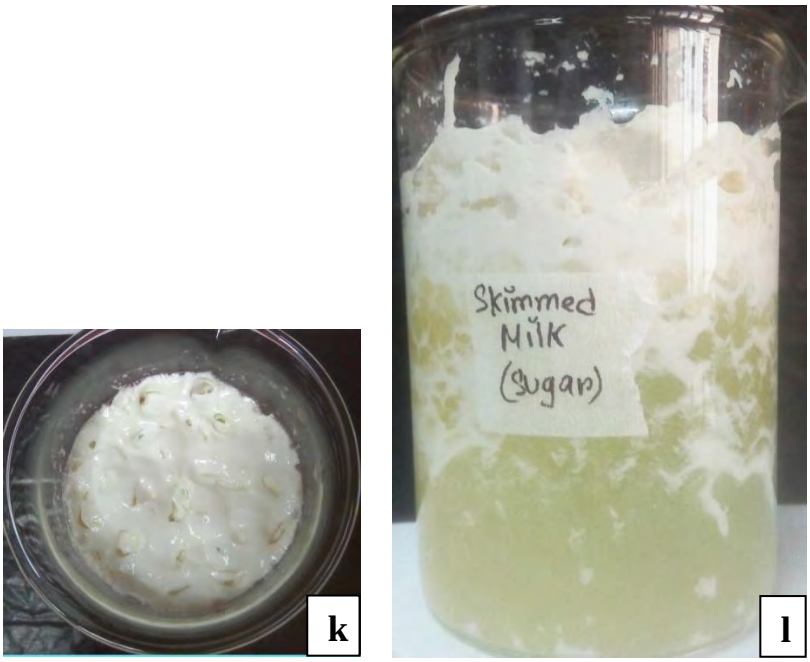


Fig.3.10: (k) Yogurt formation with single isolate in sugar added skimmed milk (top view),

(l) Side view

Table 3.4: Table demonstrating the texture, amount of water formation, taste and smell of the resulted yogurt:

Types of milk		Texture	Water formation	Color	Taste	Smell
1.	Whole milk (no sugar)	Soft and creamy	Little water	White	Sour (like yogurt)	Pungent
2.	Whole milk (sugar)	Soft and creamy	No water	White	Sweet and sour	Pungent
3.	Powdered milk (no sugar)	Semi-solid	No water	White	Sour (like yogurt)	Pungent
4.	Powdered milk (sugar)	Semi-solid and sticky	Little water	White	Sweet and sour	Pungent
5.	Skimmed milk (no sugar)	Elastic with pores	The maximum amount of water	Off-white	Salty (like cheese)	Cheesy
6.	Skimmed milk (sugar)	Elastic with pores	The maximum amount of water	Off-white	Salty (like cheese)	Cheesy

3.4.2 Dehydration of produced yogurt:

The six newly prepared yogurt samples were separately spread on aluminum foil and dehydrated at 65 ºC. Incubation resulted in drying of the thinly layered yogurt on the foils. A sterile spatula was used to extract the yogurt from all the foils and stored in sterile micro-centrifuged tubes. However, not all the yogurt samples produced a fine powder. Yogurt from skimmed milk samples did not give any dehydrated yogurt powder after drying.

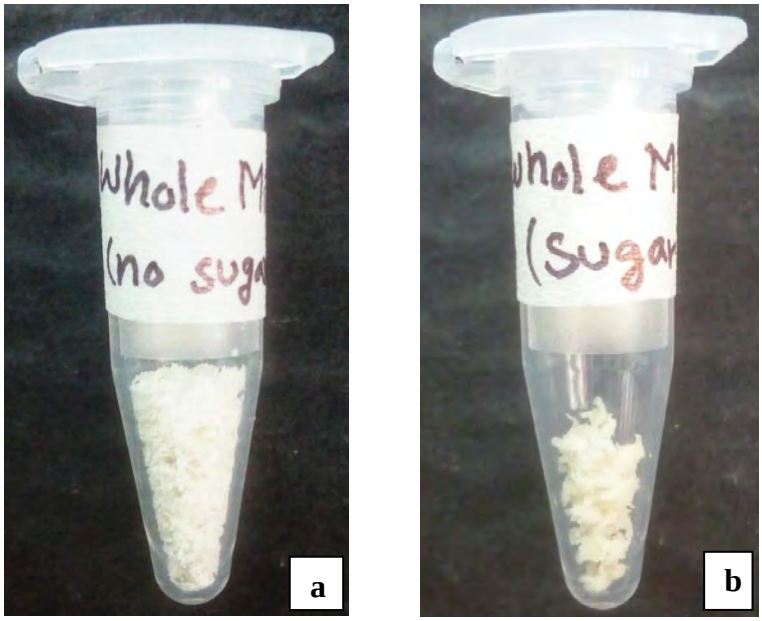


Fig. 3.11: (a) Dehydrated yogurt powder derived from whole milk,

(b) Yogurt powder derived from sugar added whole milk

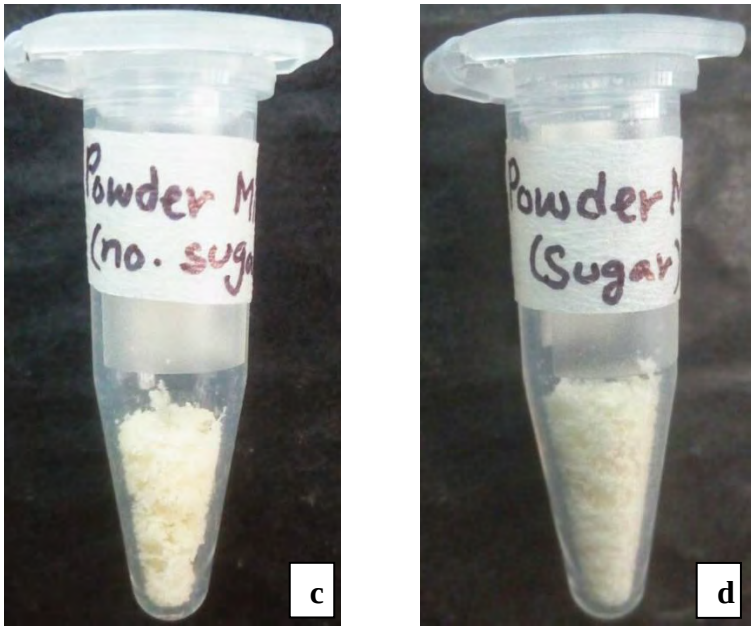


Fig. 3.12: (c) Dehydrated yogurt powder derived from powdered milk,

(d) Yogurt powder derived from sugar added powdered milk

Table 3.5: Characteristics of dehydrated yogurt:

Types of milk		Nature of dehydrated yogurt
1.	Whole milk (no sugar)	Fine powder
2.	Whole milk (sugar)	Fine powder
3.	Powdered milk (no sugar)	Fine powder
4.	Powdered (sugar)	Granule like powder
5.	Skimmed milk (no sugar)	Large granulated particles
6.	Skimmed milk (sugar)	No powder formed

3.4.3 Production of yogurt from dehydrated cells:

For this experiment, 0.25 gm from each of the dehydrated yogurt samples was inoculated into 50 ml of whole milk and powdered milk samples. In total, four beakers were prepared where two beakers had added sugar. After incubation, yogurt formation from dehydrated cells was satisfactory for whole milk.

Table 3.6: Characteristics of yogurt from dehydrated cell

Types of milk	Texture	Water formation
Whole milk (no sugar)	Soft and creamy	Little water
Whole milk (sugar)	Soft and creamy	Little water
Powdered milk (no sugar)	Almost liquid	Maximum water
Powdered milk (sugar)	Almost liquid	Maximum water

(a)

(b) **(c)**

Fig. 3.13: Formation of yogurt in whole milk from yogurt dehydrated cell. (a) Whole milk yogurt samples (side view), (b) Whole milk yogurt (no sugar) (top view) and (c) Whole milk yogurt (sugar) (top view).

(d)

(e) **(f)**

Fig. 3.14: Formation of yogurt in powdered milk from yogurt dehydrated cell. (d) Powdered milk yogurt samples (side view), (e) Powdered milk yogurt (no sugar) (top view), and (f) Powdered milk yogurt (sugar) (top view).

3.4.4 Mixed culture yogurt:

For preparing mix culture yogurt with the 10 LAB isolates, only whole milk was targeted. 50 ml whole milk (without sugar) was inoculated with 10 lactic acid bacteria and incubated. Incubation caused yogurt production through fermentation of mix starter culture of the LAB.

Here, the yogurt derived from this mixed culture was dehydrated like the previous method and mixed cultured yogurt powder was obtained. When these dehydrated cells were incubated into whole milk, it again regenerated a soft and creamy textured probiotic yogurt.

Fig.3.15: Mixed culture yogurt derived from dehydrated cells of mixed starter culture

3.5 Yogurt formation using Commercial Probiotics:

The commercial probiotics were also used for formulating new yogurts. In this experiment, 50 ml of whole milk was taken for each of the 5 previous commercial probiotics. From all the probiotics 0.25 gm was taken for inoculation into 50 ml of milk. Incubation outcome showed yogurt formation of different types for 5 probiotics. The yogurts came out with different texture and composition. The figure below shows the results.

(a) Top view

(b) Side view

Fig.3.16: Yogurt production using the commercial probiotics

Table 3.7: Features of yogurt made from commercial probiotics

Name of the commercial probiotic		Texture	Water formation	Color
1.	Probio	Soft and creamy	No water	White
2.	TS6	Elastic with pores	The high amount of water	White
3.	Emmi Swiss Yogurt	Semi-solid	Little water	White
4.	Prokids	Elastic with pores	The high amount of water	Off-white
5.	Prolacto	Elastic with pores	The high amount of water	Off-white

Chapter 4:
Discussions

4. Discussions

The present study was an ongoing project that involved much information from the previous studies. This study started with the search for Lactic acid bacteria with antagonistic properties that can be used for human benefit. As mentioned before, the objective of the present study was to justify the claims of previous projects that identified the presence of antimicrobial activities of lactic acid bacteria isolated from vegetables and dairy products. Additionally, this study has also focused on the formulation of a new probiotic yogurt starter culture that may consist of antimicrobial properties when manufactured. Besides, the study has verified the antimicrobial potentialities of some commonly available commercial probiotics. These aims have been fulfilled through continuous laboratory works to check the antimicrobial potency of LAB strains that were derived from vegetables and dairy products.

First of all, LAB strains were sub-cultured from the stocks of earlier projects. The stock isolates of the LAB were stored in T1N1 media at room temperature. Through regular sub-culture after every 3 weeks, the satisfied growth of the LAB strains was obtained. However, the previous study worked with 20 isolates but this study has used only 10 isolates. The growth of LAB from the stocks was relatively less than before. The stocks were preserved for one month before starting this present study. Storing stocks might have affected the growth of the LAB strains. This might be a reason behind that can affect the considerable growth number to get decreased. In a research experiment it has been discussed that lactic acid bacteria grow more quickly with better activity when they are present in food products (Diop et al., 2008). It is because they are biologically well adapted to the conditions of the food environment. If the strains remain separated from the food environment for a long time, their growing capability may hamper. Besides, different parameters have been used to allow maximum growth and finally isolates from 10 sources were obtained to be used for the experiments.

After the selection of 10 isolates, the antimicrobial screenings were started. These assays involved agar well diffusion method and measuring pH. In the agar well diffusion study, isolates from Aarong Dairy Sour Curd, Dhaka Cheese, Brinjal, Carrot, Turnip, Cabbage and Cauliflower showed a zone of inhibition (ZOI) against different tested pathogenic organisms. The tested organisms were *Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus pneumoniae, Bacillus subtilis, Bacillus cereus, Shigella dysenteriae, Shigella flexneri, Klebsiella sp.,*

Enterobacter cloacae, and *Salmonella typhi.* Zone of inhibition indicates the presence of some kind of antimicrobial properties might be present in LAB strains from these sources. However, the ZOI was comparatively less than the earlier studies that involved the same antimicrobial assays. As mentioned before, a poor growth environment might be responsible for the strains for not working efficiently like before. Again, in the present study, the zones of inhibition might be the result of some antimicrobial genes present in them. It was still not confirmed for which antimicrobial agents the ZOI has been created. It can be assumed that there might be the existence of genes like Bacteriocin which is nearly common for most of the lactic acid bacterium. Some possible types of Bacteriocin genes are Enterocin, Plantaricin, Pediocin, Nisin, etc. Presence of such antimicrobial genes can only be ensured through Polymerase Chain Reaction (PCR) and further genome sequencing.

Next, for measuring pH the LAB cell-free supernatants (CFS) were used. Separately pH of these samples was taken with pH meter. It was observed that the pH of the LAB CFS was 4.6-5.9. Low pH (or high lactic acid) is frequently growth limiting for lactic acid bacteria that are grown in milk or in weakly buffered bacteriological media. *Streptococci*, *Lactococci*, and other lactic acid bacteria generally grow and remain viable within a medium pH range of 4.5 to 7.0 (Hutkins and Nannen, 1993). However, the normal growth of pathogenic strains favors pH that is above 3. They cannot grow at a pH that is lower than 2-2.5. Survival of bacteria like *Escherichia coli* and *Helicobacter pylori* have been proven to be diminished at pHs that was less than 3.5, whereas killing of these strains required a pH of less than 2.5 (Jhu H et al., 2006). On the contrary, the pHs of the LAB isolates obtained from this study were not in the range of 2-3.5. Therefore, there might be some different characteristics of the LAB that were inhibiting the tested organisms in the agar well diffusion experiments. There are many pieces of evidence reporting many other secretory antibacterial components produced by LAB having a broad range of activity against Gram-positive and Gram-negative organisms which are independent of lactic acid and hydrogen peroxide (Pithva et al., 2011).

Next, this study involved some commercial probiotics to evaluate and compare their probiotic actions against pathogens. Some commonly available probiotics were chosen for this purpose from the local pharmacies of Dhaka city. This evaluation involved agar well diffusion technique

where the wells were filled with the cell-free supernatants of probiotics grown in MRS broth. The 5 commercial probiotics were: Prokids Probiotic, Prolacto, Probio, TS6 Probiotic Plus and Emmi Swiss Premium Yogurt. Nevertheless, these probiotics did not show any ZOI on MHA plates against indicator organisms *Salmonella typhi, Shigella dysenteriae, Shigella flexneri, Bacillus subtilis* and *Klebsiella pneumoniae.* There can be so many reasons for this activity. Commercial probiotics are often having many inconsistencies and deviations from the information provided on the product label, which is surprisingly very common. Frequently strains are misidentified and misclassified and products are occasionally contaminated. Sometimes this involves contamination with even facultative or obligatory pathogens. The products with such contaminations can never provide any antimicrobial efficacy against pathogens. Again, strains inside the probiotics may not be viable and the labeled number of colonies may not be verified. For these reasons, the functional properties may become diminished to the extent that can preclude the proposed health benefit (Kolacek et al., 2017).

Cross streaking was another method to check the antimicrobial potency of the commercial probiotics. In this method, the probiotic isolates were incubated with MRS broth that has been used against the same 5 tested organisms. However, no zones of inhibition were observed. It can happen because of not having an accurate number of viable cells in the total content. In Japan, the "fermented milk and lactic acid bacteria association" have developed a standard which requires a minimum of 10^7 CFU mL-1 viable the probiotic cells to be present in commercial probiotic products (Tamime et al., 2005). This may be due to the losses during storage period in which probiotics, especially *L. acidophilus*, lose their viability dramatically over the storage time and by the 20th day of storage, the viable counts of the cells decreased by at least three logarithmic cycles (Allgeyer et al., 2010). For this probiotics should be stored in its best possible way before consumption.

Bacteriocin-like inhibitory substance (BLIS) mediated antagonism is the key property of LAB strains that can be used for biopreservation in food. One can also assure a healthy gut and intestine by consuming the biologically preserved supplementary food products. Use of these food adjuncts is increasing extensively worldwide. Keeping that in mind, this study has focused on formulating a **probiotic yogurt starter culture** that will contain LAB strains with some

expected antagonistic attributes. A yogurt starter is a properly balanced blend of beneficial bacteria that will become active by consuming lactose. This mixture of bacteria will convert the lactose in milk to lactic acid giving rise to a well-textured yogurt. For this purpose, the study targeted 3 types of milk for preparing the probiotic yogurt starter culture: Whole milk, powdered milk and skimmed milk. First of all, inoculums were prepared with all the 3 types of milk in presence of and without sugar using isolates of a single source Brinjal. With the help of inoculums, 3 types of milk samples were inoculated and kept for incubation. After incubation, it was seen that the quality of produced yogurt for sugarless whole milk was the finest among the others. Its texture was soft, creamy, white colored, with a little water and had a sour pungent taste like sour yogurts. This good quality yogurt was produced because of the correct temperature (42 ºC) and incubation timing (overnight). Besides, the addition of sugar in alternate samples made the milk samples a bit solid. Like in the case of powdered milk, the sugarless milk sample was softer than the sugar added sample. It can be said that, both of the powdered milk samples had a criterion of yogurt. The final type of milk sample was skimmed milk which was basically fat-free. Usually, skim milk tends to have a slightly watery flavor. When they were inoculated with LAB isolates, it was found that after incubation both of the milk samples (sugarless and sugar added) became solidified and turned to give a cheese-like texture. The water content in these two samples was at the highest level. The samples also smelled actual cheese. The texture was elastic with multiple visible pores inside that suggests the formation of a cheese-like substance. This occurrence might have developed due to over incubation for this sample; however, the same parameters were followed for the other two types of milk. This has been proven in the initial step of making yogurt starter culture that skimmed milk should not be applicable for this preparation.

Next, the resulted yogurt samples were dehydrated at 65 ºC to extract the yogurt powder, which can rebuild the yogurt formulations. Dehydrated cells were only obtained from 2 whole milk and 2 powdered milk samples. It was not literally possible for the solidified skimmed milk samples to turn out into a powdered form. On the other hand, it was also noticed that dehydration caused the sugarless milk samples to produce more fine powder than the sugar added milk samples. So it can be concluded that, presence sugar is not permitting the formation of fine yogurt powder.

This time the dehydrated yogurt powders were inoculated into sugar added and sugarless whole milk and powdered milk. Incubation caused yogurt formation in all the 4 samples. The dehydrated cells were successfully able to form yogurt into 2 whole milk samples with a little water formation. On the contrary, the powdered milk samples could not restore the previous yogurt texture and were still in a liquid milk form even after incubation. Therefore, this can be presumed that the whole milk sample would be applicable for all of the above experiments. Whole milk can only be used for constructing the probiotic yogurt starter culture formulations.

The same procedures were followed to make yogurt and dehydrated cells from yogurt by using mixed cultures. The 10 LAB isolates were inoculated into whole milk to form yogurt and later to form dehydrated cells. The dehydrated cells from the mix culture yogurt were also able to convert milk into sour yogurt.

The next target of this study was to check the potentiality of the commercial probiotics to convert milk samples into yogurt through fermentation. For this, whole milk samples were inoculated with the 5 above-mentioned probiotics and left for incubation keeping all the parameters same as before. It was found that, the only probiotics called 'Probio' has created a creamy textured yogurt with a very less water content. Rest of the 4 probiotics caused a solidified texture like cheese in the milk with high water content. Therefore, the formation of yogurt or some solidified cheese like substances may suggest the presence of active lactic acid bacterial strains in the probiotics that unfortunately could not show any antimicrobial aspects.

Commercial benefits and Future prospects:

Up to this part the preparation of a Probiotic Yogurt Starter Culture that can make yogurt from whole milk has been discussed. At the initial phase of this study, it was also mentioned that the yogurt starter culture has to be an 'economically affordable' product to the consumers. This objective came to an attention after the market study which showed us the regular price of available yogurt products in the local markets of Bangladesh. It was observed that the price of 1kg of yogurt is around 250-350 Taka. Even 500 gm of yogurt costs 100 Taka. Now if we talk about consuming commercial probiotics for our health benefits then we should also look at their prices. The available probiotics found in Dhaka city are of different ranges. Probiotic capsules

‘Probio’ and ‘Prolacto’ were used in this study which costs 10-15 Taka per piece. Probiotics found in sachets used in the study were ‘Prokids’ and ‘TS6 Probiotic Plus’ costs 50 Taka per sachet. Again, a probiotic yogurt called Emmi Swiss Premium Yogurt has also tested whose price is 168 Taka per cup. Despite these, the commercial probiotics can hardly hold the capacity to contribute to health benefits. People in a developing country like Bangladesh are less likely interested to spent much money on such aspects. In this situation, an economically affordable as well as a probiotic property offering product will be accepted to the general mass.

During the experiments of this study, it was known that only whole milk was giving desirable results in all cases. Whole milk is easily available, cheap and results in the best quality yogurt and dehydrated yogurt powder. Furthermore, it was observed that the powdered milk and skimmed milk can hardly give satisfactory yogurt texture after incubation. That is why, powdered milk and skimmed milk were not used in rest of the experiments.

According to the aim of this study, the raw materials of the yogurt starter culture must be reasonably priced to make it affordable to its consumers. Keeping that in mind, the study has chosen the raw whole milk best for making the probiotic yogurt starter culture. Similarly, it should be noted that the cost of 1litre of whole milk is 65 Taka. The initial experiment started with inoculating 200 ml of milk samples with LAB isolates. Incubation resulted in yogurt formation in 200 ml of milk and produced approximately 200 gm of yogurt. Now, every time for making dehydrated yogurt powder, 2 gm of yogurt was spread over aluminum foil for drying. Here, we can calculate the cost of 2 gm≈2 ml of milk which is around 0.13 Taka only. Two grams of yogurt have produced 0.25 gm of yogurt dehydrated cells. Again, when this 0.25 gm of dry cells were inoculated into milk samples, after incubation yogurt were generated. Later it was assumed that, if for 200 ml of milk, 0.25 gm of yogurt starter culture was required, then for 1litre of milk it should require 1.25 gm of the dried cells. To prove this, another simple experiment was performed where 0.25 gm of yogurt starter culture was inoculated into 1litre of milk and incubated. Surprisingly, it was found that only 0.25 gm of the dehydrating cells has converted 1litre of milk into 1kg of yogurt. 0.25 gm of the dried cells was also inoculated into 50 ml of milk and the result was 50 gm of yogurt. This proves that whatever be the quantity of milk we use, 0.25 gm of the yogurt starter culture will be enough to cause fermentation after overnight incubation.

If we compare the regular price of market available 1kg yogurt with the experimental cost of this laboratory made yogurt, it can be understood that the price of the market available 1kg yogurt is 250-350 Taka, whereas this laboratory made probiotic yogurt starter culture costs 65.13 Taka only in total. Moreover, the consumers can be benefited with the nutrition of probiotics and will have to pay even less than 100 Taka.

Like the commercially available probiotics, this laboratory made probiotic yogurt starter culture can also be manufactured industrially. This study can preferably be suitable for industrial applications. To elaborate, the industrial manufacturing procedures for any product involves many other factors that may increase the total cost of the product. After the calculation of our experimental cost, it comes out that 0.25 gm of yogurt starter culture costs 0.13 Taka. Now, other costs must be added with this including the packaging cost and profits. Besides, there are always some quality conservation issues that must also be incorporated including the cost it requires to maintain. To assure the quality, these kinds of the product should be manufactured aseptically. There are some additional factors to ensure the good quality of probiotic products. This includes:

- The process should ensure that the probiotic content as mentioned on the label meets the actual content throughout the shelf life of the product, while no contamination is present.
- Probiotics have to be present in a sufficient number within the product by the end of its shelf-life, to pass through the gastrointestinal tract resisting acid and alkaline environment and to colonize the gut in a sufficient number required for exerting a measurable beneficial effect.
- The quality of the final product depends strongly on the manufacturing processes such as fermentation, matrix composition, cell harvesting, spray-drying, freeze-drying, and storage conditions like temperature, humidity, and pH are many of the manufacturing determinants that can affect microbial survival, growth, viability, and ultimately the study results and clinical outcomes.
- The identity of the microorganism at the strain level is also a prerequisite requirement to ensure that the commercial product will deliver the claimed beneficial health effect (Selim and Haider, 2014).
- The products should also be stored at the best storage conditions to avoid outside moisture and oxygen (Corcoran et al 2004).

Besides, there are some additional elements to be considered in case of competing with the other products in the market. As there are also similar products available in the market, this product needs to get better attention than the other challenging products. This may include adding popular flavors and attracting the consumers with its interesting packaging and advertisements. From this it can be inferred that, the price of 0.25 gm of the Probiotic Yogurt Starter Culture may become 5 to 10 Taka if sold in a sachet. This price is surely affordable to general mass and a new product like this will be popular for its promised benefits. In the year 2006, Food and Agriculture Organization/World Health Organization has issued recommendations on the information that should be present on the probiotic product label: genus, species, and strain designation; minimum viable number of each probiotic strain at the end of the shelf life; the suggested serving size that must deliver the effective dose of probiotics related to the health claim; proper storage conditions; corporate contact details for consumer information (Kolacek et al., 2017). If these criteria are maintained, the biofunctional probiotic yogurt starter culture designed from this study can offer good health, nutrition and diet with some of its possible antimicrobial properties and could be widely produced as native industrial products with native probiotic strains that can contribute to enhancing health in the society.

Conclusion:

The search for Lactic acid bacteria with antagonistic properties was always been very common for many researchers for the sake of human benefit. A lactic acid bacterium not only helps in fermenting food products but also takes a good care of the human gut. Bacteriocin-like substances from lactic acid bacteria are of importance in bioconservation of various foods. Moreover, the use of more than one LAB bacteriocin as a combination of biopreservatives may have major applications in improving food safety. Recently it has been discovered that probiotics have an effect on the anticancer agent. Therefore, some future studies should be performed to use these isolates reliably including molecular techniques like 16S rRNA sequencing for accurate identification of lactic acid bacterial species and multiplex RAPD-PCR technique could be used to reveal the complete metabolic potential of each of the probiotic strain which opens future research works to study for better efficacy and advancement of food biotechnological research in the food and dairy industries (Forhad et al**.,** 2015). The present study did not focus on molecular detection to identify the actual antimicrobial agent. It is possible for LAB isolates to have different inhibitory actions against pathogens that came out in the results. The advantage of this study is that the products tested were purchased independent of the companies, eliminating the conflict of interest. New probiotic products in developing countries should be manufactured with great importance because in the past decades there has been considerable interest in probiotics due to their additional health benefits. Consumer interest in healthy lifestyle and health-promoting natural products is a major driving force for the increasing global demand for biofunctional probiotics (Daniel M. Linares et al., 2017). Thus, the use of probiotics should be further investigated for both possible benefits and side-effects in people affected by different medical conditions.

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Appendix I

Media compositions

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium Chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

MRS Agar (oxoid)

Component	Amount (g/L)
Peptone	10.0
Lab-Lemco Powder	8.0
Yeast Extract	4.0
Glucose	20.0
Di-potassium hydrogen phosphate	2.0
Agar	10.0
Sodium acetate $3H_2O$	5.0
Tri-ammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

T_1N_1

Component	Amount (g/L)
Tryptone	1.0
Sodium chloride	1.0
Agar	0.75

Mueller-Hinton Agar

Component	Amount (g/L)
Beef, infusion	300.0
Casamino acids	17.5
Starch	1.5
Agar	17.0

Lactobacillus MRS Broth

Component	**Amount (g/L)**
Dextrose	20.0
Protease peptone	10.0
Beef extract	10.0
Yeast extract	5.00
Sodium acetate	5.00
Ammonium citrate	2.00
Dipotassium phosphate	2.00

Appendix II

Instruments

Instrument Manufacturer	Instrument Manufacturer
Electric Balance	Scout, SC4010 USA
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Water Bath	Daihan Scientific Companies, Korea
Table Top Centrifuge	Digisystem, Taiwan
Microscope	A. Krüssoptronic, Germany
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Microcentrifuge tubes	Tarsons Products, Pvt Ltd, Kolkata