Isolation of Lactic Acid Bacteria with Antimicrobial Activity

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

Submitted by

Sihinta Sabeen Shembil

Student ID: 11336003

August, 2016

Department of Mathematics and Natural Sciences

Biotechnology Programme

BRAC University

Dhaka, Bangladesh

DECLARATION

I hereby declare that the research work representing the results reported in this thesis paper entitled **"Isolation of Lactic Acid Bacteria with Antimicrobial Activity"** submitted by Sihinta Sabeen Shembil, has been carried out under the supervision of **Dr. M. Mahboob Hossain**, Coordinator, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. Moreover, it is also to be declared that the research work presented here is original, and has not been submitted to other institutions for any degree or diploma.

Sihinta Sabeen Shembil
Candidate

Certified:

Dr. M. Mahboob Hossain
Coordinator and Associate Professor,
Microbiology Program
Department of Mathematics and natural Sciences
BRAC University
Dhaka, Bangladesh

Dedicated to my loving Grandfather, my dear Dada Shamsul Haq, for his continuous support and blessings

Acknowledgement

First and foremost, I am grateful to the Almighty for giving me the means to complete my research work.

I offer my sincere gratitude to **Prof. A. A. Ziauddin Ahmad**, Chairperson, Department of Mathematics and Natural Sciences, BRAC University and **Prof. Naiyyum Choudhury**, Coordinator of Biotechnology and Microbiology program, BRAC University for giving me access to the laboratory and research facilities.

I would like to express my deepest admiration to my supervisor, **Dr. M. Mahboob Hossain** Coordinator, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. I never hesitated to ask him for help and I am immensely thankful for his ongoing support, helpful insights and encouragement throughout my thesis research.

I can never thank Salman Khan Promon, Teaching Assistant, enough for introducing me to my thesis project and giving me guidance. I value all the help he has given me for it saved me a lot of time and frustration, and also for the innovative techniques that made me get the more out of my thesis project.

I would like to give my thanks to Tasmin Naila, former Teaching Assistant, for giving me several demonstrations on laboratory techniques along with all the helpful advice.

I am happy to have had someone as knowledgeable as Kieran Williames give me support. He was like a source of light within the darkness. He explained to me great details in simple terms. My thesis project benefited immeasurably through the mentoring he gave me in both theoretical and practical aspects.

I was fortunate to have had Dipanjanna Datta to work alongside me during the practical part of my thesis. My time spent during this thesis project was improved with her lively moments

infused with sarcasm, which served as a much needed source of humor. I am happy we crawled out of the dim tunnel together.

Moreover, I will always be grateful towards the Department of English and Humanities, BRAC University, for giving me balance.

Lastly, I could not have completed my research project if it weren't for Asma Binte Afzal, Lab officer of MNS Department and Nahreen Mirza, Teaching Assistant, for sharing their skills and knowledge during my work. I am also grateful for all the rest of the laboratory Teaching Assistants, who were always there to give invaluable advice.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

LAB: Lactic Acid Bacteria

MRS: (de Man, Rogosa and Sharpe)

ºC: Degree Celsius

et al: and others

FAO: Food and Agriculture Organization of the United Nations

WHO: World Health Organization

GRAS: Generally Recognized As Safe

FDA: Food and Drug Administration

spp: Species

Da: Dalton

STGG: Skim Milk-Tryptone-Glucose-Glycerol

ml: Milliliter

µl: Microliter

MH: Mueller Hinton

ZOI: Zone Of Inhibition

MIU: Motility Indole Urea

MR: Methyl Red

VP: Voges-Proskauer

MSA: Mannitol Salt Agar

TSI: Triple Sugar Iron

ABSTRACT

The benefits of live cultures as probiotics have been known for a long time throughout the world; probiotics can improve immunity, prevent diseases and much more. Probiotics can be a great substitute for antibiotics. Fortunately, Lactic Acid Bacteria (LAB) are ubiquitous in nature. In the present investigation, 35 different food samples were analyzed and of them six contained LAB. Three types of Lactic Acid Bacteria were derived from bitter gourd, brinjal, broccoli, cauliflower, maize and potato: *Streptococcus, Enterococcus* and *Lactobacillus*. The aim, with these bacteria, was to observe their antimicrobial activity as whole cells and to check if they produce bacteriocin, a high molecular weight peptide; for both these tests, it was observed if growth of six selected pathogenic strains would occur or would be inhibited in the presence of whole cells and supernatants. Gram positive and Gram negative pathogenic strains were selected: *B. subtilis, B. cereus, E.coli, S. aureus, S. flexneri* and *S. pneumoniae*. To test the antimicrobial activity of the whole cells against the selected strains, the agar overlay method was applied. Well diffusion method was used to investigate the presence of bacteriocins with antimicrobial properties; this method was done with both crude supernatant and supernatant precipitated with ammonium sulphate. The agar overlay method requires the isolate to be stabbed into MRS (de Man, Rogosa and Sharpe) agar and incubated anaerobically for 24 hours at 37° C, and then soft agar seeded with pathogen was poured evenly on top. This was incubated for another 24 hours at 37° C. After conducting the agar overlay method zones of inhibitions were present. It was seen that all of the isolates inhibited all of the pathogenic strains, except for the isolate from brinjal against *S. pneumoniae*. As for the well diffusion method, Mueller-Hinton agar requires the pathogen to be swiped on its surface and the agar to be punctured to make wells, and then the supernatant of the LAB isolates were dispensed into the wells; this was incubated for 18-24 hours at 37° C. No antimicrobial activity was observed through the well diffusion method.

Chapter 1

Introduction

1.1 Introduction

The term “probiotics” is derived from the Greek “probios”, which is defined as “for life” (Florou-Paneri *et al.*, 2013). During earlier times probiotics have been well acknowledged through fermented dairy products. Probiotics still hold great significance today as this field has been explored intensively for the past several decades. Nowadays the focus is not merely on bacteria collected from fermented dairy products but also bacteria collected from the intestines (Sanders, 1999). According to FAO/WHO, probiotics are known as live cultures of microorganisms which are beneficial for the host human or host animal. They are helpful since they improve the indigenous microflora when consumed in ample amounts (Yusuf & Hamid, 2013).

Tannock mentions that the estimated number of bacterial population in and on the human body is 100,000,000,000,000 cells; this includes areas such as the skin, oral cavity and intestinal tracts (Sanders, 1999). Through research it is assumed that these probiotic bacteria have a wide range of health effects such as ameliorating stomach and intestinal tract functions, improving immune functions and even decreasing cancer incidents (Sanders, 1999). The main benefit of probiotics is their role in preventing diseases. What is remarkable is that probiotics are capable of affecting any colonized region of the body, and the bacteria can influence the body both locally and systemically. Hence the benefits of probiotics can be respected (Council for Agricultural Science and Technology, 2007).

The common foods containing probiotic cultures are yoghurt, cheese, breakfast cereals, sausages, infant formulas and more (Pyar & Peh, 2014).Within the Bangladesh population, yoghurt remains the most well-known and largely consumed probiotic food (Hoque *et al.*, 2010). As there has been a grown interest in food that contain useful bioactive characteristics, According to Hugo and Antoni, lactic acid bacteria (LAB) are target organisms for their probiotic and antimicrobial properties against pathogenic microorganisms (Kazemipoor *et al.*, 2012).

1.2 Probiotics

Nowadays, the use of probiotic in Bangladesh for both humans and animals has increased. In Bangladesh there are several probiotics made available for animal production, such as Bactosac, Probac, Poultry Star sol and Gutpro. All of these products contain LAB species. According to Frost and Sullivan, in other countries like Europe, the food and beverage probiotic market has outstanding success within the food industries (Selim & Haider, 2014).

Hence it can be seen that probiotics are used in Bangladesh for animal production and it is known that dairy products such as yoghurt and cheese are commonly consumed in Bangladesh, and these are good sources of probiotics. This goes to show that LAB probiotics are promising and can be implemented in many ways in the industries of Bangladesh, which was a motivating factor for conducting this study.

1.3 Lactic Acid Bacteria

Lactic acid bacteria (LAB) have been used for fermentation and preservation of food for many years. They are known to be generally-recognized-as-safe (GRAS) by the FDA. Hence the bacteria or their cultured by-products can be used as food ingredients (Vijayakumar & Muriana, 2015). LAB is a diverse bacterial group consisting of gram-positive, aerotolerant, acid-tolerant, low GC content, usually non-sporulating and non-respiring rod or cocci microorganisms. Most of the LAB members are capable of converting lactose and other sugars to lactic acid (Chowdhury *et al.*, 2012). Fortunately LAB are easily found throughout nature. They can be located in milk and dairy products, raw meat, fermented products and vegetables.

The major genera of LAB are: *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus,* and *Enterococcus.* The peripheral genera are: *Vagococcus, Weissella, Oenococcus, Lactosphaera, Aerococcus, Bifidobacterium* and more. The LAB make up a high percentage of bacteria that provide probiotic properties (Yusuf & Hamid, 2013). This can be seen in Table 1.1.

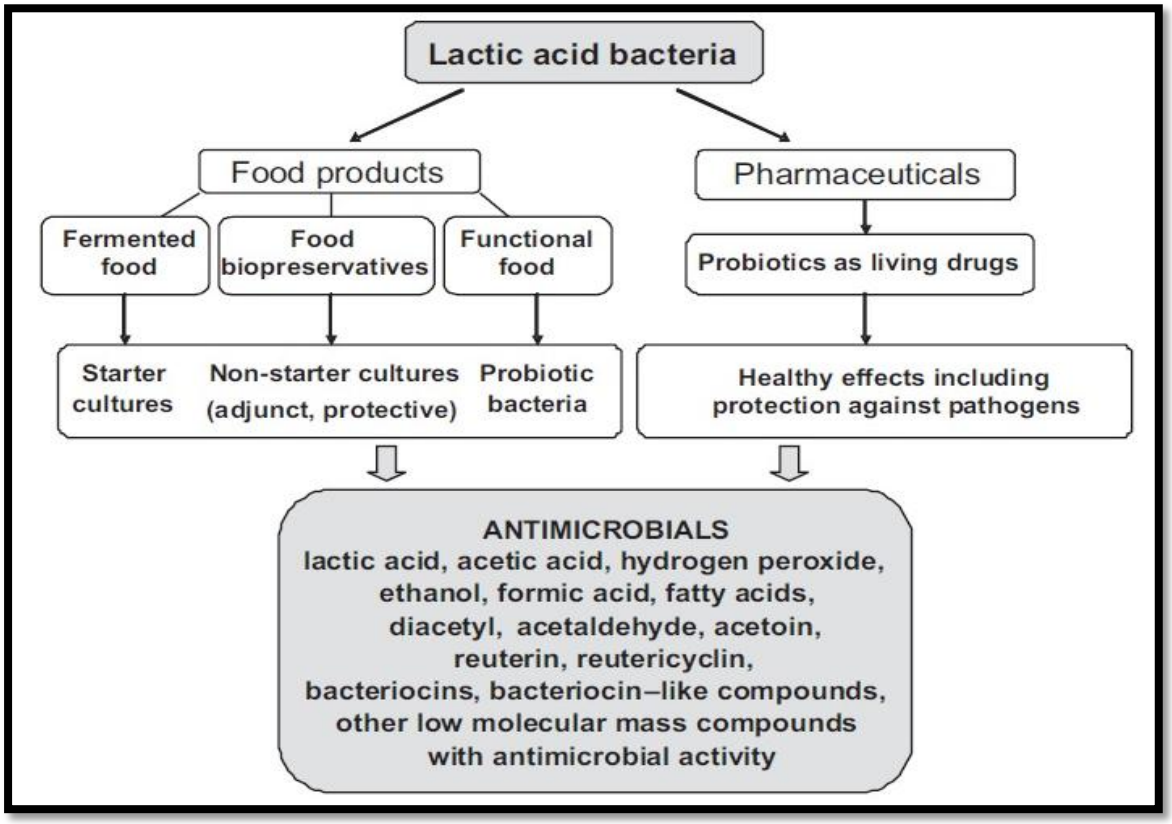
Table 1.1: Key genera species of microbes studied and used as probiotics. (Council for Agricultural Science and Technology, 2007)

Genus	Species
Lactobacillus	*acidophilus* *brevis* *fermentum* *plantarum* *reuteri*
Bifidobacterium	*Adolescentis* *bifidum* *infantis* *longum*
Streptococcus	*thermophilus* *salivarius*
Enterococcus	*faecium*

For this reason, LAB can be a vital part of certain industrial food processes because they contribute to the quality and hygiene of the products. LAB have the ability to prevent spoilage from other microorganisms as well as the growth of pathogenic bacteria through its antimicrobial metabolic products such as organic acids and bacteriocin (Kormin *et al.*, 2001).

The collection and use of LAB have been growing more significant recently due to the rise of antibiotic resistant pathogens. An alternative is required for the increased utilization of antibiotics. *Lactobacillus* spp, for example, fulfills this necessity as it can tolerate harsh conditions and at the same time produce extracellular bacteriocin, which ultimately inhibits a diverse number of pathogenic strains (Chowdhury *et al.*, 2012). Therefore, LAB are promising in terms of replacing chemical preservatives and antibiotics due to its antimicrobial compounds which, as Frederic has mentioned, may play noteworthy roles within the host, especially the human gastrointestinal tract (Adebayo *et al.*, 2014). The various sectors that LAB can contribute in is shown in figure 1.1.

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



1.4 Antimicrobial Properties of LAB

Selection of probiotics heavily depends on the antimicrobial activity that they provide. LAB give out antimicrobial effects through certain substances such as lactic acid, acetic acid, propionic acids, carbon dioxide, hydrogen peroxide, diacetyl, bacteriocins and other low molecular weight substances (Chowdhury *et al.*, 2012).

Collins, La Ragione, Woodward & Searle have pointed out that antimicrobial substances of LAB can be categorized into two different groups: low molecular mass substances (<1000 Da) and high molecular mass substances (>1000Da) (Suskovic *et al.*, 2010). High molecular mass substances refer to bacteriocins. All other antimicrobial substances fall under low molecular mass substances. The low molecular mass substances include organic acids, diacetyl, hydrogen peroxide and carbon dioxide. For the organic acids, lactic and acetic acid are best characterized. These acids and the amount of these acids products during fermentation have impact upon the microbial activity of the fermented material and is capable

of causing a toxic effect on other microbes. The activities of the main molecular mass substances are mentioned below, along with Table 1.2 (Suskovic *et al.*, 2010).

- Organic acids, which arise through conversion of sugars, are the major material in preservation because they reduce the pH. Lactic acid and Acetic acid are the most well-known organic acids of LAB. The quantities of these acids are important because their amount influence the antimicrobial effect in the fermented material.

- The impact of carbon dioxide on preserving food is great. Not only does it have its own antimicrobial traits, it also supplies an anaerobic environment as it replaces the present molecular oxygen. Carbon dioxide provides antifungal activity because it inhibits enzymatic decarboxylations and prevents it from building up in the lipid bilayer membrane, which ultimately results in permeability malfunction.

- Hydrogen peroxide is highly antimicrobial due to its strong oxidizing impact on bacterial cells; Hydrogen peroxide breaks down the basic molecular structure of cell proteins.

- 2,3-butanedione, diacetyl, is commonly known for the buttery scent it gives out in fermented dairy products. High quantities of diacetyl are needed for food preservation.

Table 1.2: Low molecular mass antimicrobial metabolites of LAB

Compound	Microorganisms Producers	Antimicrobial Spectrum
Lactic acid	All lactic acid bacteria	Yeasts Gram-positive bacteria Gram-negative bacteria
Acetic acid	Heterofermentative lactic acid bacteria	Yeasts Gram-positive bacteria Gram-negative bacteria
Diacetyl Acetaldehyde Acetoin	Variety of genera of lactic acid bacteria including: *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus*	Yeasts Gram-positive bacteria Gram-negative bacteria

Hydrogen Peroxide	All lactic acid bacteria	Yeasts Gram-positive bacteria Gram-negative bacteria
Carbon dioxide	Heterofermentative lactic acid bacteria	Most of the taxonomic groups of microorganisms

(Suskovic *et al.*, 2010).

1.4.1 Bacteriocins

Bacteriocins are bioactive proteins and peptides, with high molecular weight, that have been synthesized ribosomally. It is mentioned that bacteriocins can be bacteriostatic or bactericidal, meaning they either inhibit the growth of pathogenic microbes or they kill pathogenic microbe. Whichever method the bacteriocin applies, the bacteriocin will not harm the producer cell (Yusuf & Hamid, 2013). The production of bacteriocins by the LAB cells depends on various physical factors such as temperature, pH and source of nutrients. Additionally, bacteriocin production is the highest during the end of log phase and beginning of stationary phase, during cell growth. (Yusuf & Hamid, 2013).

Bacteriocin may have a narrower spectrum against other bacteria than antibiotics. However, bacteriocin can work alongside other metabolic products of LAB such as organic acids. Bacteriocins and antibiotics are distinguished in Table 1.3. Most LAB bacteriocins work against other bacteria by puncturing cell membranes and scattering the proton motive force. Nevertheless, gram-negative bacteria are not distressed by this fatal attack because their outer membrane provides protection (Yusuf & Hamid, 2013). Nettles and Barefoot stated that for this reason bacteriocins are identified to have antimicrobial activity towards gram positive bacteria, especially closely related species and food spoilage and pathogenic bacteria such as: *Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes* (Kormin *et al*,. 2001).

Table 1.3: Main differences between (LAB) bacteriocins and conventional antibiotics (Perez *et al.*, 2014)

Characteristic	Bacteriocins	Antibiotics
Application	Food/Clinical	Clinical
Synthesis	Ribosomal	Secondary metabolite
Bioactivity spectra	Mostly narrow	Mostly broad
Proteolytic enzyme degradability	High	Moderate-to-none
Thermal stability	High	Low
Active pH range	Wide	Narrow
Color/taste/odor	No	Yes
Amenability to bioengineering	Yes	No
Possible mechanism of target cell developing resistance	Adaptation through changes in cell membrane composition	Genetically transferable determinant that inactivates the active compound
Toxicity towards eukaryotic cells	Relatively no	Yes

Perhaps the bioactivity spectra are narrow for bacteriocins, which may seem disappointing. However, bacteriocins have many benefits over antibiotics ranging from high tolerance to heat and acidity to lower toxic effects. Due to these advantages, bacteriocins are receiving a lot of attention as they can be used as alternative therapeutics in pharmaceutical areas and also as preservation in food industries (Yusuf & Hamid, 2013).

1.5 Objectives

Probiotic bacteria are capable of inhibiting the growth of pathogenic strains because they compete for nutrition and attachment sites on the colonic epithelium, and also produce organic acids and bacteriocins (Balamurugan *et al.*, 2015). Moreover if the LAB have antagonistic effects against the pathogenic species, this would prove them as highly useful

because a large proportion of diseases arise due to popular food containing bacterial growth (Siddique *et al.*, 2012).

LAB would be an inexpensive and convenient way to overcome this, and it will impact on the health and economic system. Moreover, there are very few published works in Bangladesh regarding the effectiveness of LABs against pathogens (Siddique *et al.*, 2012). For that reason performing this study could shed some light on that, through the assessment of the antimicrobial activity of LAB whole cells and LAB extracellular material against certain pathogens. The main objectives of this study are:

- Collect LAB from various sources; sources include dairy products, fruits and vegetables from both natural sources and store-brought items.
- Discover LAB whole cells that have antimicrobial activity against pathogenic strains: *B. subtilis, B. cereus, E.coli, S. aureus, S. flexneri, S. pneumoniae*.
- Observe if the antimicrobial LAB provide antimicrobial activity through their extracellular supernatants.

Chapter 2

Materials and Methods

2.1 Location of study

This study was carried out in the Biotechnology and Microbiology Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Collection of Samples

Lactic Acid Bacteria were sourced out from vegetables, fruits dairy, herbs and others. There were 35 samples in total, as shown in table 2.1.

Table 2.1: **Samples tested to source out lactic acid bacteria**

Vegetables		**Fruits**	**Herbs/ Spices/ Others**		**Dairy**
Bitter gourd	Olives (bottled)	Dragon fruit	Aloe vera*	Maize**	Emmi curd
Brinjal	Peapods	Guava	Centella asiatica	Mushroom***	Home curd*****
Broccoli	Potato	Lychee	Coriander	Parsley	Milk (cow)
Carrot	Pumpkin	Mango	Garlic	Tofu****	Milk (human)
Cauli-flower	Ridge gourd	Pomegranate	Ginger		Pran sour curd
Cucumber	Spring onion	Dragon fruit	Green chilli		Rosh sour curd
Long beans			Holy basil		

Note: * Aloe Vera is a medicinal plant **Maize is classified as a cereal grain. *** Mushroom belongs to the fungi kingdom. ****tofu is a derivative product of soy bean. *****Home curd refers to a home-made yoghurt.

2.2.1 Bacterial growth from samples

In regards to the solid samples, growth of LAB was encouraged through fermentation. This was done by cleaning and dicing the samples before placing them in salt solution (Kazemipoor *et al.*, 2012). The liquid samples were directly added to MRS (de Man, Rogosa, Sharpe) broth and incubated for 24-72 hours at 37° C.

- The solid samples were washed thoroughly under distilled water, after being diced. Then they were placed into volumetric flasks containing 0.9% saline solution (0.9g

sodium chloride in 100 ml water). The volumetric flasks were covered tightly with aluminum foil and parafilm, to create an environment that is favorable for fermentation. The sorted samples were left for two to three weeks, in room temperature. Afterwards, 1 ml of this solution was placed into 9 ml of MRS (de Man, Rogosa and Sharpe) broth using a pipette. It was ensured that the fermented solution was collected from the bottom of the flask, because that area is most anaerobic.

- Liquid samples were used directly; 1 ml of the sample was placed into 9 ml of MRS broth using a micro-pipette.
- The inoculated MRS broths were vortexed and then incubated for 24-72 hours (until turbid) anaerobically at 37° C. After this, the broths are ready for use or can be stored at 4°C.
- To test the presence of LAB, lawns were formed on MRS agar from the incubated MRS broth. Firstly a serial dilution of x2 was prepared using 0.9% saline solution. 200 ul of this was dropped onto a MRS agar plate, and then the spread plate technique was applied. Distinct colonies were expected after 24-72 hours of anaerobic incubation at 37°C.
- If colonies appeared, then four biochemical tests were done to ensure the sources are LAB: catalase test, Gram stain, endospore stain and acid fast stain. These methods were chosen according the Bergey's Manual of determinative bacteriology.

It was concluded that no LAB was present in the samples that gave no growth of bacteria in the MRS plates. For the samples that did grow in colonies, unique colonies were selected and streaked onto fresh MRS agar plates. These plates were incubated anaerobically at 37°C for 24-72 hours.

2.3 Identifying LAB from samples

Identification of LAB was determined based on biochemical test results using Bergey's manual of determinative bacteriology. Gram positive, catalase negative, endospore negative were maintained on MRS agar plates and stored at 4°C for further tests (Adebayo *et al.*, 2014).

i) Catalase Test:

This test is simple and fast, hence this was done first on all the samples to separate the possible LAB species (catalase negative) from the non-LABs (catalase positive).

- 2-3 drops of catalase reagent (35% Hydrogen Peroxide) were placed on a clean dry glass slide.
- A bacterial colony was picked using a clean sterile tooth pick and mixed into the catalase reagent.
- Bubble formation (oxygen) within 5-10 seconds of the addition of reagent indicated positive for catalase activity.

ii) Gram stain:

All LAB species are gram positive, hence the gram staining method was conducted. Gram staining was done using standard procedure (Cappuccino & Sherman, 2005).

iii) Endospore stain:

LAB are non-spore forming bacteria, meaning they will only stain pink and show no green stains under the microscope. (Cappuccino & Sherman, 2005).

iv) Acid fast stain (Ziehl–Neelsen stain):

In Bergey's manual of determinative bacteriology, *lactobacillus* was differentiated from *Mycobacterium* through acid fast stain. *Lactobacillu*s species give a negative result (blue) for acid fast staining (Cappuccino & Sherman, 2005).

- A smear of an isolate was heat fixed on a slide and then placed over a beaker with boiling water giving off steam.
- A generous amount of carbol fuchsin stain was applied over the smear for 5 minutes and reapplied to replace evaporated stain.
- After 5 minutes the slide was cooled and gently washed with distilled water.
- Acid alcohol was used to decolorize the stain and then methylene blue was used to flood the smear for 2 minutes.
- Observation under microscope: The acid fast positive bacteria would retain pink stain from carbol fuchsin. Acid fast negative bacteria would be stained blue.

2.4 Storage and Subculture of LAB isolates

After the microscopic evaluations and catalase tests, the isolates that matched with the characteristics of LAB were kept for further experiments.

2.4.1 Subculturing of LAB onto MRS agar plates

Bacteriocin production is known to be supported in MRS media, a synthetic complex, according to previous studies (Wendawi & Saady, 2012). The collected isolates were streaked on to fresh MRS agar plates every two weeks and stored at 4°C. The four-way streak plate inoculation was followed for this experiment (Cappuccino & Sherman, 2005). The isolates were streaked on to fresh MRS agar plates every two weeks and stored at 4°C.

2.4.2 Long term storage

Each of the isolates was preserved in Skim Milk-Tryptone-Glucose-Glycerol (STGG) media, at -20°C, and also in T1N1 agar, at room temperature.

2.5 Primary screening of LAB isolates: agar overlay method

From the collected isolates, each colony was stabbed into MRS agar and incubated overnight, at 37°C, under anerobic conditions. Then the plates were overlaid with soft agar seeded with indicator strains (Kormin *et al.*, 2001). Six different indicator strains, belonging to both gram-positive and gram-negative groups, were tested: *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae* and *Shigella flexneri* (Chowdhury *et al.*, 2012). These were all laboratory strains.

- With an inoculating needle, a tiny amount of LAB was collected and stabbed into fresh MRS agar plates, where the bacteria were incubated anaerobically for 24-48 hours, at 37°C. Growth was expected to be visible but not be excessive on the surface of MRS agar.
- 10 ml of nutrient broth was inoculated with a loopful of an indicator strain. Hence there were six nutrient broths for six different pathogens. Each broth was vortexed and incubated at 37°C overnight.

- Six sets of nutrient soft agar (0.5g agar agar per 100 ml of nutrient broth) were prepared. After autoclaving, the soft agar was allowed to cool down, and a small portion of the soft agar was poured into a sterile petri dish to check for contamination and comparison.
- Then each soft agar was inoculated with a pathogen of interest (100 ul of pathogen per 2 ml of nutrient soft agar). This was vortexed to ensure proper mixing. These were also poured into a sterile petri dish to aid in comparison and check any contamination.
- A thin layer of this soft agar, containing indicator strain, was poured on top of the MRS agar stabbed with LAB. Once the soft agar had solidified, the plates were returned to the 37°C incubator for 24 hours, under aerobic conditions. Then the plates were checked for any zone of inhibitions (ZOI).

2.6 Secondary screening of LAB isolates: well diffusion

The agar well diffusion method is suitable to test for the antimicrobial property of LAB. This method requires a 24 hour culture of the pathogens (was suspended in saline), which is used to prepare a lawn of the indicator strain by spreading the cell suspension over the surface of nutrient agar plates with a sterile cotton swab. After the plates dry, wells are made in the agar using a sterile cork borer of diameter (5 mm). Each well is then filled with the culture free filtrate obtained from the LAB isolates. Antimicrobial activity from the supernatant was observed after overnight incubation at 37°C; ZOI around the wells indicate antimicrobial activity. Results were considered positive if the diameter (mm) of the ZOI was greater than 1mm (Kazemipoor *et al.*, 2012).

2.6.1 Well diffusion method to detect bacteriocin

- 10 ml MRS broths were heavily inoculated with sample isolates. The broths were vortexed and anaerobically incubated at 37°C for 24 hours.
- The MRS broth cultures were centrifuged at 14,000 x g for 20 minutes.
- The supernatants were transferred into a sterile falcon tube, and were adjusted to pH 7 using 1N NaOH (Adebayo *et al.*, 2014).
- A sterile 0.22 µm syringe filter was used to transfer the pH adjusted fluids into a new sterile falcon tube. The supernatant should not be mixed with the pellet.

- The six indicator strains were transferred into 0.9% saline and vortexed. The turbidity matched with 0.5 MacFarlene.
- The saline solutions were used to prepare lawns on Mueller-Hinton (MH) agar plates. For each indicator strain a sterile cotton swab was used. Afterwards, with a sterile cork-borer, 5mm wells were punctured into the MH agar plate (Nowroozi *et al.*, 2004). There was one well for each sample and an extra well for the control. The punctured gel was removed with the aid of a sterile inoculating needle.
- Using a micropipette 50 µl of each sample's supernatant was added to a well, and 50 µl of uncultured MRS broth was added to the control well. It is important to fill up the wells within 15 minutes of spread-plating with the indicator strains.
- The MH plates were placed at 4°C for 2 hours and then incubated overnight at 37°C.
- Observation for any ZOI that is 1mm or greater was conducted.

2.6.2 Ammonium sulphate precipitation well diffusion

The well diffusion protocol was repeated, but with ammonium sulphate precipitation, instead of merely cell free supernatant. The centrifuged supernatant was further purified through treatment with 80% ammonium sulphate at 4ºC. Then bacteriocin precipitate was extracted by centrifugation (Adebayo *et al.*, 2014).

- The same method above was followed, except addition of ammonium sulphate (8 g per 10 ml) to the cell free supernatant fluids was done and then the samples were refrigerated at 4 ºC overnight.
- Centrifugation at 14,000 x g for 20 minutes was completed. Most of the supernatant was discarded, only a small amount was left to dissolve the pellet.
- Well diffusion was conducted in the same manner as mentioned above, with the pellet-containing fluid.

2.7 Biochemical tests to identify genus of LAB isolates

The LAB screened for antimicrobial activity were identified based on the following characteristics.

1. Microscopic observations

2. Biochemical reactions

Microscopic observations include the tests mentioned previously: Gram stain, Endospore stain and acid fast stain.

2.7.1 Biochemical Tests

Several biochemical tests were carried out to identify the bacteria with the aid of Microbiology Laboratory Manual (Cappuccino & Sherman, 2005). The bacteria tested were grown on nutrient agar plates at 37ºC in the incubator.

i) Carbohydrate fermentation tests: arabinose, fructose, galactose, glucose, lactose, sucrose, maltose, and mannitol

- Labeled test tubes were filled with a carbohydrate broth: (arabinose, fructose, galactose, glucose, lactose, sucrose, maltose and mannitol).
- Inverted durham tubes were placed in the glucose broth, and they were fully filled with the broth.
- Each tube was aseptically inoculated with pure bacterial culture from MRS agar plates. Then the tubes were incubated for 24 hours at 37ºC.
- The result was interpreted by observing change of colours.

 Change of colour from red to yellow: The change of the colour of broth to yellow indicated acid formation from fermentation with or without gas production.
 No colour change: If the colour remained read it meant absence of fermentation.
 Gas production: The presence of bubbles in the inverted durham tube indicated gas production accompanied by acid production or alcohol fermentation.

ii) Casein hydrolysis test

- Distilled water was collected, according to the needed amount, and divided into two conical flasks. Nutrient agar was dissolved into one. Both the conical flasks, one with dissolved nutrient agar and the other with distilled water, was autoclaved.

- Skim milk agar was mixed intp the autoclaved distilled water once it had slightly cooled.
- Then the two solutions were mixed together and it was ensured that no lumps were present. After this, the solution was poured into sterile petri dishes.
- The samples were inoculated into the agar in a zig-zag manner. Then they were incubated at 37ºC for 24 hours. Observation for any clear areas around the organism growth was conducted.

 Clear zones: clear zones around the growth of the organisms indicate casein hydrolysis.
No clear zones: If the agar appears opaque around the grown organism, then this means the organism does not hydrolyze casein.

iii) Citrate utilization test

- Simmon Citrate agar medium was used to test for citrate utilization. The bacteria from a pure culture were streaked on the slant of the agar in a tube.
- The change in colour was observed after an incubation period of 24 hours at 37ºC.
- In a positive reaction, the color of the medium turned blue. No change in color indicated negative result for citrate utilization.

vi) Gelatin hydrolysis test

- Medium was prepared by dissolving 5.0g peptone, 3.0g beef extract and 120g liquid gelatin per litre.
- 2 to 3 ml of the medium was dispensed into test tubes, and autoclaved.
- The media was inoculated with an inoculating needles before being incubated at 37ºC for 48 hours.
- The cultures were placed in the refrigerator, 4ºC for 30 minutes, and then rapid liquefaction of gelatin was checked.

- The solidified cultures were returned to the incubator for another five days. Afterwards, the cultures were kept at 4ºC for 30 minutes and liquefaction of gelatin was checked again.

 Rapid liquefaction: If media liquefies at 4ºC for within 48 hours, that means the organisms produce gelatinase and demonstrate rapid gelatin hydrolysis

 Liquefaction: If media liquefies at 4ºC after five days, that means the organisms are capable of producing gelatinase.

 No liquefaction: : If media is solid at 4ºC, then this indicates that organisms do not produce gelatinase.

v) Growth at 45 ºC

- Organisms were inoculated onto MRS plates in a zig-zag fashion.
- These plates were incubated at 45 ºC for 24-48 hours.
- If growth appears, then that means the organisms are thermophiles. If growth does not appear, than that means that 45 ºC and higher temperature is not suitable for the organism's growth.

vi) Hemolysis test

- Blood agar was streaked with bacterial isolate and incubated for 24 hours
- The result was interpreted as follows.

 Alpha-hemolysis: This was indicated by presence of brown-green discoloration under the bacterial growth

 Beta- hemolysis: This was observed from the clearing of red color from the agar around the bacteria due to breakdown of RBC in the agar.

 Gamma-hemolysis: The lack of discoloration or clearing of medium indicated gamma- hemolytic bacteria (negative for hemolysis).

vii) Motility, urease activity and indole production test

- A motility indole urea (MIU) semisolid medium was used to determine motility, indole production and urease activity of the bacteria. Fresh bacteria from a pure culture were used to inoculate with a needle. The needle was stabbed 2/3rd way of the medium in the test tube approximately at the centre.
- The organisms were also inoculated in tryptophan broth to check for indole.
- The medium was kept at 37ºC in an incubator for 24 hours.
- Kovac's reagent was added to tryptophan broth.
- The growth and result was interpreted as follows.

 Motility: The bacteria were considered positive for motility if there was turbid growth spreading from the stab line in the MIU media.

 Indole production: Kovac's reagent was added at the end of incubation. Appearance of cherry red reagent layer indicated positive reaction for indole production while negative reaction was considered if the layer remains yellow or brown.

 Urease activity: Positive urease activity was signified by the change of color in the MIU media to pink from orange due to the production of ammonia after incubation.

viii) Lipid hydrolysis

- Tributyrin agar plates were inoculated with organisms in a zig-zag pattern.
- The plates were incubated at 37 ºC for 24-48 hours.
- Results were recorded upon observation of clear areas around the growth of the organisms. Clear areas indicate lipolysis, whereas absence of clear areas indicate no lipolysis.

ix) MR-VP (Methyl Red - Voges-Proskauer) test

- Potassium phosphate broth (MR-VP broth) containing dextrose, peptone and potassium phosphate was inoculated by a loopful of pure bacterial isolate and incubated at 37ºC for 24 hours.
- The broth (6 ml) was divided equally among two tubes to perform MR (methyl red) test and VP (Voges-Proskauer) test.

MR test reaction:

- Bacteria may have produced acid through the incubation period to suppress the phosphate buffer and make the broth acidic. Methyl red was added and the test was considered as positive if red colour formed.

VP test reaction:

- Barritt's reagent A was added to an already incubated potassium phosphate broth and shaken slightly.
- Barritt's reagent B was added in equal amounts to reagent A. The test tube was kept still for 15 minutes.
- Appearance of a red colour on the reagent layer signified positive reaction.

x) Mannitol Salt Agar (MSA) test

- Organisms were streaked on to MSA plates in a zig-zag pattern.
- Plates were incubated at 37 ºC for 24-48 hours.
- If mannitol fermentation occurs, than this means the organism is mannitol fermentor.

xi) Nitrate reduction test

- Nitrate broth was inoculated with loopful of bacterial isolate and incubated for 24 hours.
- Nitrate reduction test reagents A and B were added in equal amounts after incubation period.
- The observation of colour change to a deep red within a few moments of addition of reagents A and B indicated presence of nitrate reductase enzyme.
- Zinc powder was added if no colour change was observed after addition of reagents A and B. The appearance of red colour after this step confirmed a negative result for nitrate reduction. Colourless solution after zinc powder addition indicated presence of both nitrate reductase and nitrite reductase.

xii) Oxidase test

- A Whatman filter paper (1mm) was soaked with the oxidase reagent.
- A loopful of pure culture bacteria was streaked on it.
- Within 1 – 30 seconds, the appearance of purple color over the bacteria is a positive result. Delayed reactions are ignored and concluded as negative.

xiii) Starch hydrolysis test

- Bacteria from a pure culture was streaked on a starch agar plate along a straight line and kept for incubation for 24 hours at 37ºC.
- After incubation, iodine solution was dropped over the growth.
- A positive result was indicated by clearing of the media surrounding the bacterial growth.

xiv) Triple sugar iron (TSI) fermentation

- Using an incolulating needle, organism was picked up and then stabbed into slanted TSI agar's butt. The needle was withdrawn and streaked over the slant's surface. The lid was loosely closed to ensure entrance of air.
- The TSI agar was incubated at 37ºC for 24 hours.
- Results was recorded according to information below.

 Sugar fermentations:

Acid butt, alkaline slant (yellow butt, red slant): Glucose has been fermented but not sucrose or lactose.
Acid butt, acid slant (yellow butt, yellow slant): Lactose and/or sucrose has been fermented.

Alkaline butt, alkaline slant (red butt, red slant): Glucose, lactose, nor sucrose has been fermented.

 Gas production: Indicated by bubbles in the butt. With large amounts of gas, the agar may be broken or pushed upward.

 Hydrogen sulfide production: Hydrogen sulfide production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of H_2S with the ferrous ammonium sulfate to form black ferrous sulfide. The black precipitate indicates that the bacteria were able to produce hydrogen sulfide (H_2S) from sodium thiosulfate. Because H_2S is colorless, ferric ammonium citrate is used as an indicator resulting in the formation of insoluble ferrous sulfide. Formation of H_2S requires an acidic environment; even though a yellow butt cannot be seen because of the black precipitate, the butt is acidic.

Chapter 3

Results

3.1 Growth and identification of Lactic Acid Bacteria (LAB)

Six isolates, from the 35 samples, were tested as LAB: Bitter gourd, Brinjal, Broccoli, Cauliflower, Maize and Potato.

Table 3.1: Growth of sample isolates and LAB on MRS agar

Sample	MRS growth	LAB	Sample	MRS growth	LAB	Sample	MRS growth	LAB
Aloe vera	+ve	-ve	**Green chilli**	+ve	-ve	Coriander	-ve	
Broccoli	+ve	+ve	**Guava**	+ve	-ve	**Maize**	+ve	+ve
Carrot	+ve	-ve	**Lychee**	+ve	-ve	**Mushroom**	+ve	-ve
Cauliflower	+ve	+ve	**Long beans**	-ve		**Parsley**	+ve	-ve
Garlic	+ve	-ve	**Mango (unripe)**	-ve		**Tofu**	+ve	-ve
Ginger	+ve	-ve	**Olives**	-ve		**Emmi curd**	+ve	-ve
Potato	+ve	+ve	**Pea pods**	+ve	-ve	**Home curd**	+ve	-ve
Spring Onion	+ve	-ve	**Pomegranate**	+ve	-ve	**Milk (cow)**	+ve	-ve
Bitter gourd	+ve	+ve	**Pumpkin**	+ve	-ve	**Milk (human)**	+ve	-ve
Brinjal	+ve	+ve	**Ridge gourd**	+ve	-ve	**Pran sour curd**	+ve	-ve
Cucumber	+ve	-ve	**Centella.**	-ve	-ve	**Rosh sour curd**	+ve	-ve
Dragon fruit	+ve	-ve	**Holy basil**	-ve	-ve			

note: shaded samples were further studied in this experiment.

On MRS agar plates, colonies of LAB grew and matured within 24 to 48 hours. These colonies were smooth, round, opaque, cream in colour and had smooth edges. Gram's staining, spore staining, acid fast staining and catalase tests were done on colonies to differentiate the LAB isolates from the non-LAB isolates. Morphology was also viewed under a compound microscope at 100X.

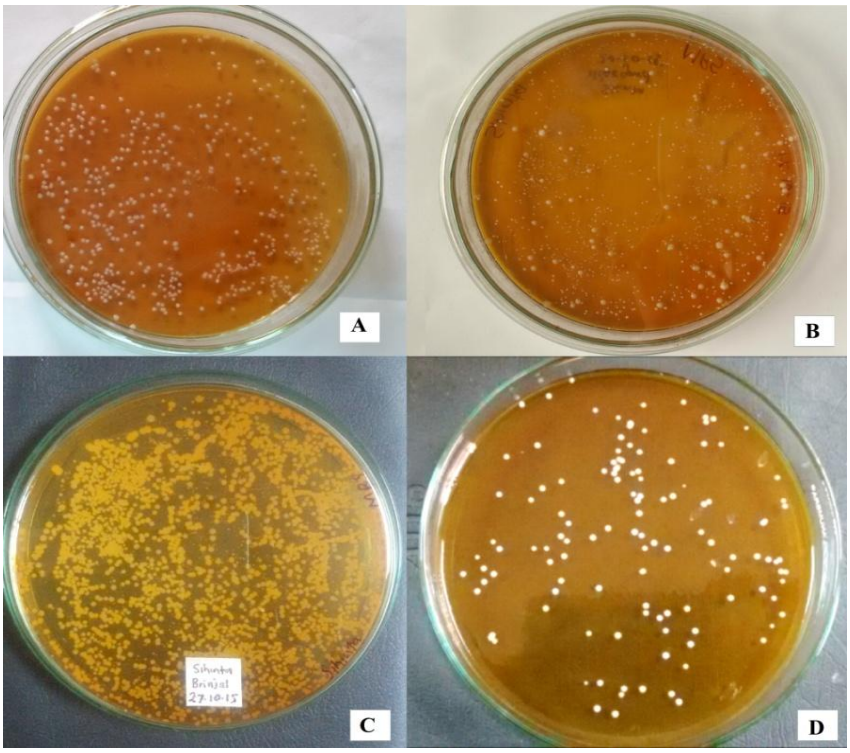


Figure 3.1: Growth of bacteria on MRS agar from spread-plate technique. Isolates were collected from A: Cauliflower B: Maize C: Brinjal, D: Broccoli

Table 3.2: Gram staining, catalase, spore staining, acid fast staining and morphology results

Isolate Source	Gram's Staining	Catalase	Spore Staining	Acid Fast Staining	Morphology
Bitter Gourd	+ve	-ve	-ve	-ve	Rod
Brinjal	+ve	-ve	-ve	-ve	Coccus
Broccoli	+ve	-ve	-ve	-ve	Coccus
Cauliflower	+ve	-ve	-ve	-ve	Coccus
Maize	+ve	-ve	-ve	-ve	Coccus
Potato	+ve	-ve	-ve	-ve	Rod

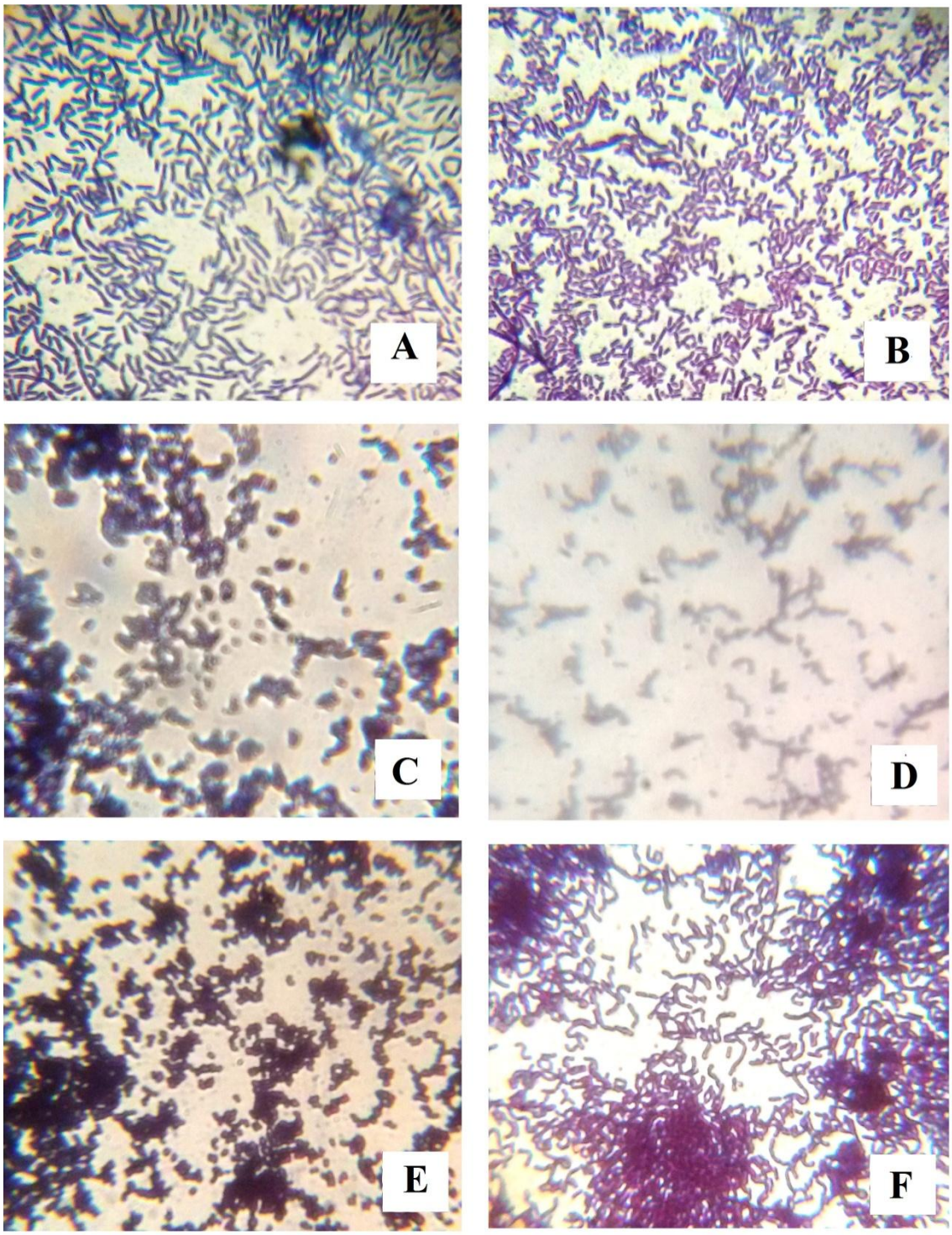


Figure 3.2: Microscopic observations of the isolates after gram staining. All results show the isolates to be gram-positive. Isolates were collected from A: Bitter gourd B: Brinjal C: Broccoli D: Cauliflower E: Maize F: Potato. Potato and Bitter gourd isolates are rod-shaped. The other four isolates are cocci.

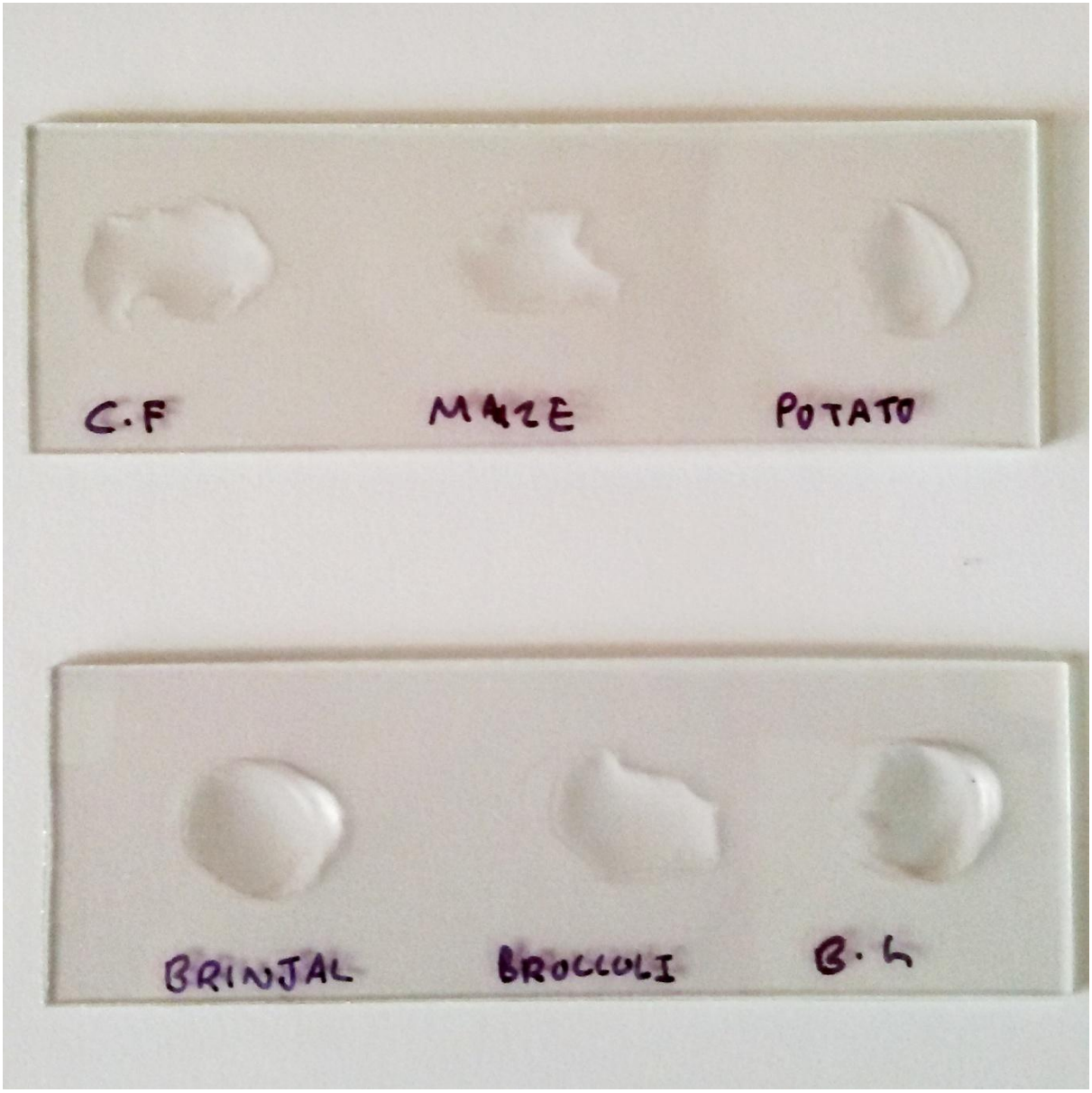


Figure 3.3: Results of catalase test (35% hydrogen peroxide) showed that each of the six isolates is catalase-negative, as no bubble formation occurred.

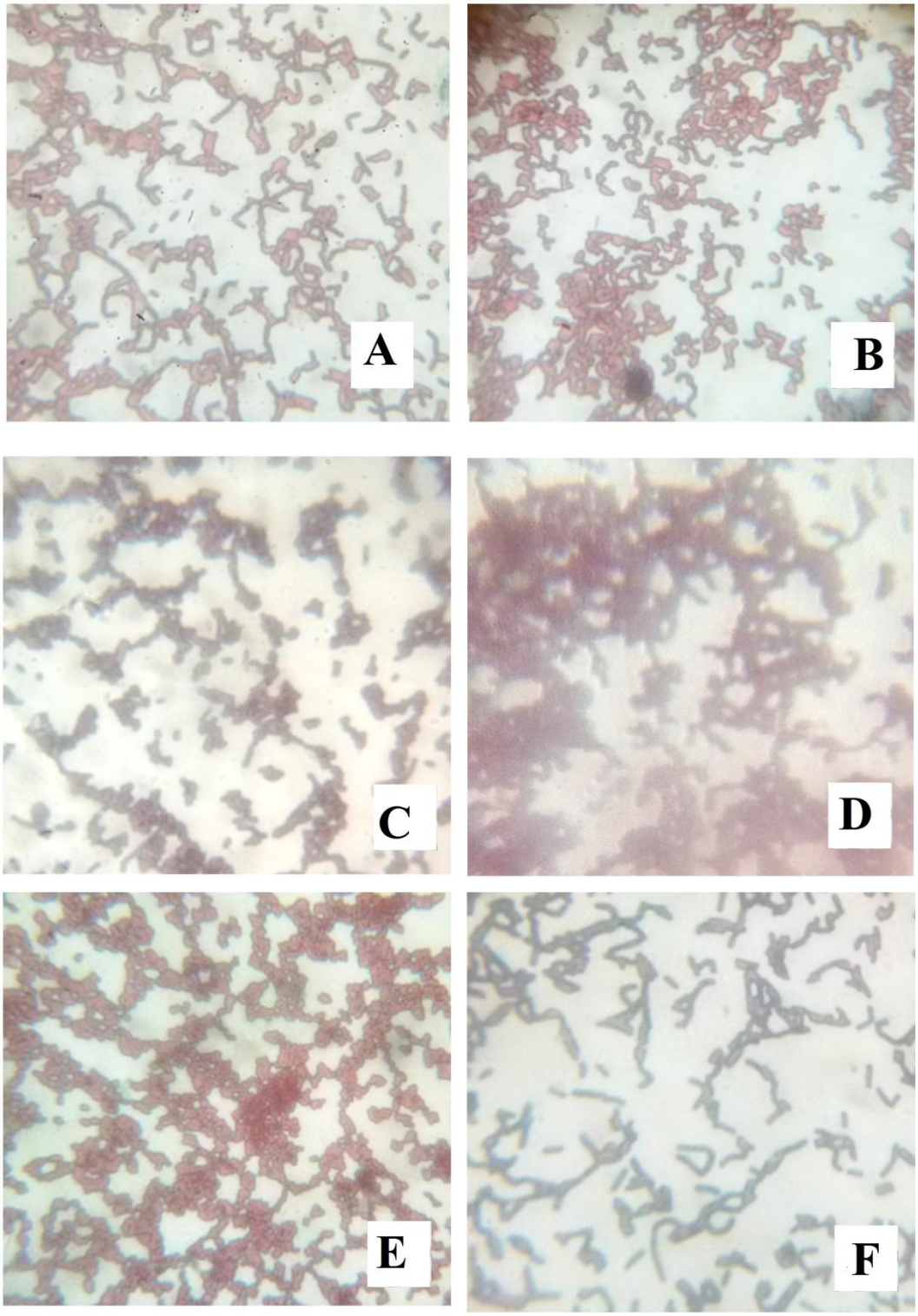


Figure 3.4: Microscopic observations of the isolates after spore staining. The cells did not retain any green colour hence the results indicate that all the isolates lack endospores. Isolates were collected from A: Bitter gourd B: Brinjal C: Broccoli D: Cauliflower E: Maize F: Potato.

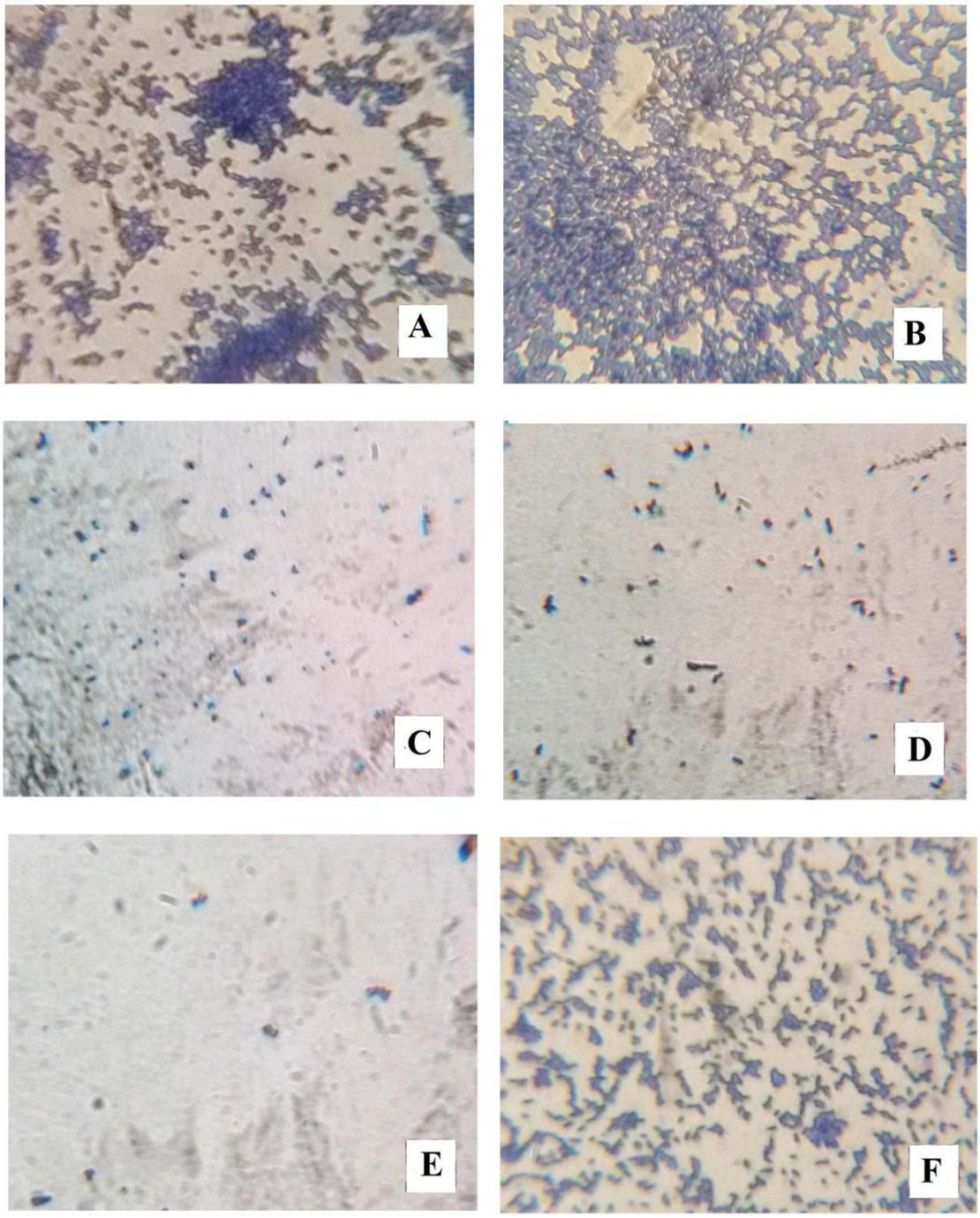


Figure 3.5: Microscopic observations of each of the six isolates, after acid fast staining, show only blue colour. This means all the results are negative. Isolates were collected from A: Bitter gourd B: Brinjal C: Broccoli D: Cauliflower E: Maize F: Potato.

3.2.1 Primary Screening: Agar Overlay

Antagonist effect of the six isolates was tested against six pathogens. All the isolates gave ZOI against all the indicator strains, except from Brinjal against *S. pnuemoniae.*

Table 3.3: Zone of inhibition observed through agar overlay method of the six isolates against the six pathogens

	Isolates from					
	Bitter gourd	**Brinjal**	**Broccoli**	**Cauli-flower**	**Maize**	**Potato**
B.cereus	✔	✔	✔	✔	✔	✔
B. subtilis	✔	✔	✔	✔	✔	✔
E.coli	✔	✔	✔	✔	✔	✔
S. aureus	✔	✔	✔	✔	✔	✔
S. flexneri	✔	✔	✔	✔	✔	✔
S. pneumoniae	✔	x	✔	✔	✔	✔

✔ = Positive x = No effect

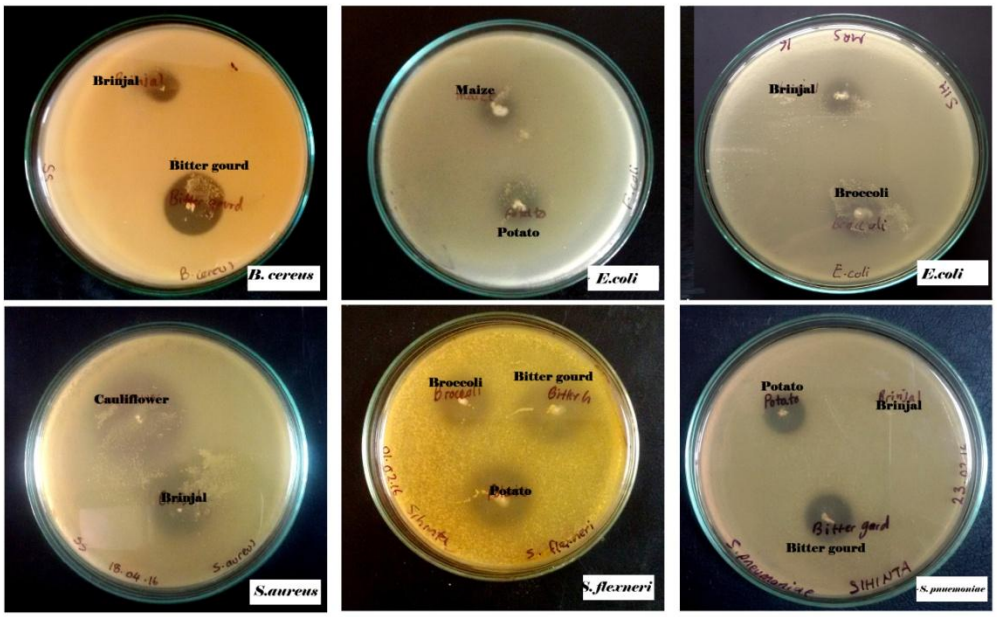


Figure 3.6: Zone of inhibition formation from agar overlay method

3.2.2 Secondary Screening: Well diffusion

The secondary screening, which involved the well diffusion method, was conducted with both the crude supernatant and the precipitated cell-free supernatant collected from the six isolates. The supernatants were tested against the six different pathogens. No ZOI was observed.

Table 3.4: ZOI observation from well diffusion method of the six isolates against the six pathogens

	Isolates from					
	Bitter gourd	**Brinjal**	**Broccoli**	**Cauli-flower**	**Maize**	**Potato**
B.cereus	x	x	x	x	x	x
B. subtilis	x	x	x	x	x	x
E.coli	x	x	x	x	x	x
S. aureus	x	x	x	x	x	x
S. flexneri	x	x	x	x	x	x
S. pneumoniae	x	x	x	x	x	x

x = No effect

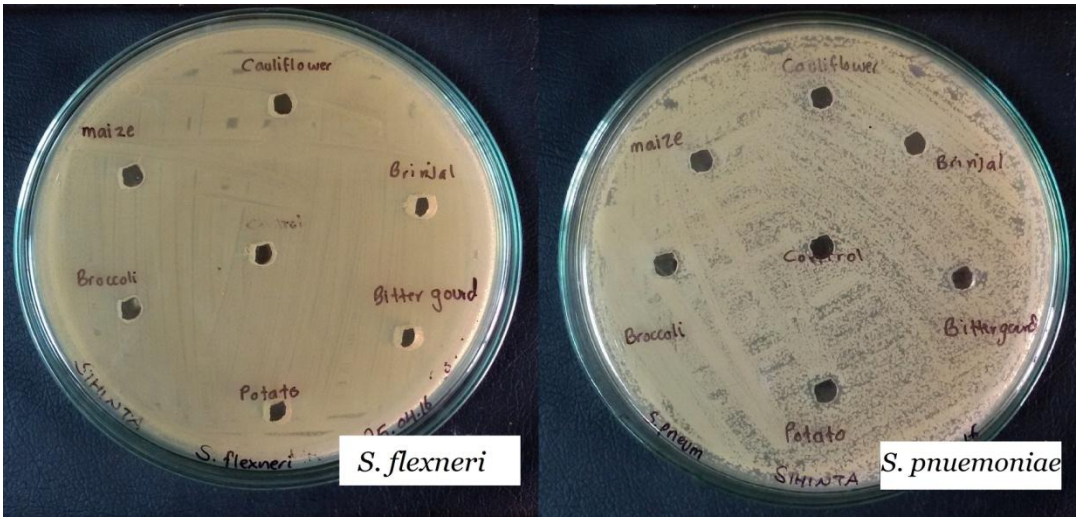


Figure 3.7: Results of well diffusion MH-agar plates after 16-24 hours incubation. No zones of inhibition are present. Left: the six isolates against *S. flexneri*. Right: the six isolates against *S. pnuemoniae*

3.3 Identification of bacterial isolates

Table 3.5: Biochemical test results of the six isolates

Isolate source	Casein hydrolysis	Citrate utilization	Gelatin hydrolysis	Lipid hydrolysis	Hemolysis	Nitrate reduction	Indole production	Urease	Motility	Oxidase	Growth on MSA	Growth at 45°C	H_2S Production	Methyl-Red test	Voges-Proskauer test	Facultative anaerobe	Fermentation Tests									Presumptive organism
																	Glucose	Glucose with acid	Fructose	Sucrose	Maltose	Lactose	Galactose	Arabinose	Mannitol	
Bitter Gourd	-ve	-ve	-ve	-ve	γ	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	*Lactobacillus delbrueckii*
Brinjal	+ve	-ve	+ve	-ve	γ	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	*Enterococcus* spp. or *Streptococcus* spp.
Broccoli	-ve	-ve	-ve	-ve	γ	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	*Enterococcus* spp. or *Streptococcus* spp.
Cauli-flower	-ve	-ve	-ve	-ve	β	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Streptococcus* spp.
Maize	-ve	-ve	-ve	-ve	γ	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	*Enterococcus* spp. or *Streptococcus* spp.
Potato	-ve	-ve	-ve	-ve	β	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Lactobacillus casei*

Chapter 4

Discussion

4.1 Discussion

In this study, six out of the 35 samples were confirmed to be lactic acid bacteria species (gram positive, catalase negative, endospore negative and acid-stain negative). Further biochemical tests showed that the six isolates were *Streptococcus, Enterococcus* and *Lactobacillus*, according to the flowchart from Bergey's Manual of Determinative Bacteriology.

In the primary screening, which involved the agar overlay method, all of the isolates gave zones of inhibition against all of the pathogens, except that from brinjal against *S. pneumoniae.* According to the results of primary screening, the LAB isolate as whole cells have strong antimicrobial effects against the indicator strains.

The secondary screening method, which consisted of well diffusion with mere supernatant and precipitated supernatant, yielded no positive results. No ZOI was observed for any of the isolates against any of the tested pathogens. From this, it can be said that the supernatant does not contain antimicrobial activity at a detectable level. Furthermore, because bacteriocins are extracellular material, it can be suggested that the LAB isolates either do not contain or contain too low concentration of bacteriocin to inhibit the growth of the tested pathogens. Overall, the LAB isolates contain antimicrobial activity as whole cells.

Bacteriocin activity is more preferable for antimicrobial properties, however LAB that can provide any addition inhibition of pathogens is also desirable (Vijayakumar & Muriana, 2015). For this reason, the isolates from this study can be paired with other LAB isolates that do contain bacteriocin activity, because it can provide enhanced antimicrobial effects.

Majority of authors recommended MRS (de Man, Rogosa and Sharpe) media for the growth of LAB. However, to enhance bacteriocin production, a few alterations can be made. For example, adding peptone, reducing sodium chloride levels (0.5%), increasing glucose and fructose levels and ensuring no starch is present can allow MRS media to influence bacteriocin production *(*Al-Wendawi & Al-Saady, 2012).

Other studies conducted on bacteriocin-containing LAB have given positive results against selected pathogens. However, it must be pointed out that these studies had a larger quantity of samples to begin with. One study, focused on raw cattle milk, and was able to obtain 10 strains that gave positive results out of a total of 100 strains (Mohankumar & Murugalatha, 2011). A different experiment sourced out 25 LAB from appam dosa batter, and from these 25 isolates 10 gave positive results (Pal *et al.*, 2005). Another study included LAB from retail food and animal samples; the study began with 170 food samples and 110 animal samples. From the food samples, 41 isolates detected *L. monocytogenes*. As for the animal samples, only 11 of the isolates detected *L. monocytogenes*. Hence, 14 percent of the food samples and 10 percent of the animal samples gave positive results (Henning *et al.*, 2015). Furthermore, in a study that aimed to find bacteriocin producing LAB in meat found that 174 out of 285 meat sources contained LAB. 813 colonies were collected from the 174 samples, and 128 colonies gave positive results. This can also be calculated as 15.7 percent of colonies that contained bacteriocins (Bromberg *et al.*,, 2004). Finally, one study involved gastrointestinal tracts from six chickens; 307 LAB strains were collected and no more than 14 strains proved to be bacteriocin producing strains (Musikasang *et al.*, 2012). From these conducted studies, it can be seen that only a small proportion of the entire sample sources contained the target material: bacteriocin. Therefore, since this study itself contained 35 samples and merely six LAB isolates, the chance of finding a bacteriocin-producer was small.

4.2 Limitations:

LAB are ubiquitous in nature, but only six LAB isolates were found from 35 samples. More LAB isolates were expected from the total number of samples.

Previous experiments have shown that many bacteriocins are produced by *Lactobacillus* and some by *Bifidobacterium (*Al-Wendawi & Al-Saady, 2012). For this reason, more *Lactobacilli* species were desired but only two out of the six isolates were *Lactobacilli*.

Inhibitory effects were found against pathogens when the whole cells were used. This tells us that the isolates have anti-microbial properties but it does not tell us which parts are actually responsible for the antimicrobial activity. It could be due to a wide range of different substances, or it could even be due to different substances working together. Metabolites such

as lactic acid, acetic acid, hydrogen peroxide and so forth could be responsible but we cannot be certain which ones are and which ones are not. Without knowing this, we cannot know the mode of inhibition either. For example, acids inhibit growth by lowering pH whereas hydrogen peroxide inhibits growth by denaturing enzymes. Knowing which substances are accountable is useful because then the particular substance can be extracted and manipulated to see if they work against pathogens on their own.

Another drawback is the possibility of whole cells containing substances that could result in harmful side effects for the consumer. However, this is unlikely because LAB are recognized as GRAS. Hence species identification, not merely genus identification, is needed to confirm further safety.

4.3 Further research and Recommendations:

The results of this study would be more fruitful if a larger and more diverse sample was tried out, as this could increase the chance of locating bacteriocin-producing LAB isolates. This experiment only focused samples from products fit for human consumption, but LAB species can be located in water bodies, soil, humans and animals. Despite LAB having antimicrobial properties as whole cells, bacteriocins are far more preferable as they can replace antibiotics in a healthier manner.

Since studies have shown that majority of bacteriocin for LAB species were extracted from *Lactobacillus* species, the aim should be to focus on locating sources with *Lactobacillus* species.

The isolates collected can be added to commercial products such as probiotics, cheese and yoghurt. This would increase the industrial value of the LAB microorganisms. If one food item had to be the focus, then it should be yoghurt because yogurt is highly enriched, natural, supports growth and survival of probiotics, and is favourable to many (Nikkhah, 2014). Moreover, dairy products such as cheese and yogurt are commonly eaten by the population of Bangladesh, by people of both higher and lower classes. Therefore adding the LAB isolates into these food products could benefit the overall society.

For the LAB to work effectively within the host, the LAB must survive within the host's body. One problem is resisting inhibitory factors in the gastrointestinal tract such as bile salts. To ensure the LAB can withstand this factor, different concentrations of bile salts should be added to the MRS broth (Pyar & Peh, 2014).

The genus of the LAB isolates have been determined in this study, however knowing which species the isolates are is essential too. The information derived from sequence data will provide prospects to enhance probiotic functionality and also to increase understanding of mechanisms. (Sanders, 1999).

To enhance bacteriocin production, the media and incubation parameters can be altered. In previous studies, maximum production of bacteriocin occurred MRS broth containing 1-2% glucose or xylose. Along with this, MRS medium with 1% NaCl showed that antimicrobial activity increased. Bacteriocin production follows a growth curve, and the inhibitory activity has shown to be at its peak at the beginning of the stationary phase (Nowroozi *et al.*, 2012). The growth curve of bacteriocin can be monitored by incubating the same samples for different periods of time, such as a 24 hour sample up to a 72 hour sample.

If bacteriocin activity occurs in the secondary screening, well diffusion method, then one more examination should be completed to see if the bacteriocin has bacteriocidal or bacteriostatic effects: using a sterile cotton swab, swipe over the ZOI on the well diffusion agar plate. Then streak the cotton swab over a fresh nutrient agar plate, and place in the incubator for 24 to 72 hours. If growth occurs, that means the LAB supernatant inhibited growth of the pathogen (bacteriostatic). However if no growth appears than it means the LAB supernatant killed the pathogen (bactericidal).

Recommendations for future work:

1. Test more samples from a variety of different sources: soil, tap water, human milk, dental cavities, meat, animal faeces and more. The focus should also be on *Lactobacilli* over other LAB species.
2. Identify species through PCR procedure.

3. Changes to MRS medium can be made to see if it enhances the growth of LAB and bacteriocins.
4. Investigate when bacteriocin production is highest on the growth curve by using 24, 48 and72 hour old broths, and compare.
5. In this study, *enterococcus* species and *streptococcus* species were not differentiated. Differentiation should be done by conducting the bile eschulin biochemical test.
6. Perform bacteriocin isolation using ammonium sulphate and a dialysis membrane.
7. Use more pathogenic strains to increase information on which kinds of pathogens the LAB isolates work against.
8. Add compounds, such as bile salts, that would potentially act as inhibitory factors within the host's body, to check in the isolates can withstand it.
9. Antibacterial activity can be further characterized by determining whether it is bacteriostatic or bactericidal.
10. Incorporate the isolated LAB species into industrial food products.

REFERENCES

Adebayo, F., Afolabi, O., & Akintokun, A. (2014). Antimicrobial properties of purified bacteriocins produced from Lactobacillus casei and Lactobacillus fermentum against selected pathogenic microorganisms. British Journal of Medicine and Medical Research, 4(18), 3415-3426.

Al-Wendawi, S. A., & Al-Saady, A. A. (2012). Screening for Bacteriocins Production in Enteric Bifidobacterium Isolates and Study of Some Production Affecting Factors. Medical Journal of Babylon, 9(2), 387-394.

Balamurugan, R., Chandragunasekaran, A. S., Chellappan, G., Rajaram, K., Ramamoorthi, G., & Ramakrishna, B. S. (2014). Probiotic potential of lactic acid bacteria present in home made curd in southern India. Indian Journal of Medical Research, 140(9), 346-348.

Bromberg, R., Moreno, I., Zaganini, C., Delboni, R. R., & De Oliveira, J. (2004). Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. Brazilian Journal of Microbiology, 35(1-2), 137-140.

Cappuccino, J., & Sherman, N. (2005). Microbiology: A Laboratory Manual. Peason new international edition.

Chowdhury, A. (2012). Screening of Lactobacillus spp. from Buffalo Yoghurt for Probiotic and Antibacterial Activity. Journal of Bacteriology & Parasitology, 3(8), 1-3.

Florou-Paneri, P. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes, 589-593.

Henning, C., Vijayakumar, P., Adhikari, R., Jagannathan, B., Gautam, D., & Muriana, P. (2015). Isolation and Taxonomic Identity of Bacteriocin-Producing Lactic Acid Bacteria from Retail Foods and Animal Sources. Microorganisms, 3(1), 80-90.

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology. European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society.

Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S., Billah, M. M., & Islam, K. M. (2010). Isolation , Identification and Analysis of Probiotic Properties of Lactobacillus Spp . From Selective Regional Yoghurts. World Journal of Dairy & Food Sciences, 5(1), 39-45.

Kazemipoor, M., Radzi, C. W., Begum, K., & Yaze, I. (2012). Screening of antibacterial activity of lactic acid bacteria isolated from fermented vegetables against food borne pathogens. Archives Des Sciences, 65(6), 192-197.

Kormin, S., Rusul, G., Radu, S., & Ling, F. H. (2001). Bacteriocin-producing lactic Acid bacteria isolated from traditional fermented food. The Malaysian Journal of Medical Sciences : MJMS,8(1), 63-67.

Mohankumar, A., & Murugalatha, N. (2011). Characterization and Antibacterial Activity of Bacteriocin Producing Lactobacillus Isolated from Raw Cattle Milk Sample. International Journal of Biology, 3(3), 128-130.

Musikasang, H., Sohsomboon, N., Tani, A., & Maneerat, S. (2012). Bacteriocin-producing lactic acid bacteria as a probiotic potential from Thai indigenous chickens. Czech Journal of Animal Science, 57(3), 137-143.

Nikkhah, A. (2014). Probiotics & Health Yogurt the Most Natural and Healthy Probiotic : History Reveals. Journal of Probiotics and Health, 2(2), 1.

Nowroozi, J., Mirzali, M., & Norouzi, M. (2004). Study of Lactobacillus as Probiotic Bacteria.Iranian Journal of Public Health, 33(2), 1-5.

Pal, V., Jamuna, M., & Jeevaratnam, K. (2005). Isolation And Characterization Of Bacteriocin Producing Lactic Acid Bacteria From A South Indian Special Dosa (Appam) Batter. Journal of Culture Collections, 4, 53-55.

Perez, R. H., Zendo, T., & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): Various structures and applications. Microbial Cell Factories,13(1), 2.

Pyar, H., & Peh, K. K. (2014). Characterization and identification of Lactobacillus Acidophilus using biolog rapid identification system. International Journal of Pharmacy and Pharmaceutical Sciences, 6(1), 189-192.

Sanders, M. E. (1999). Probiotics. SCIENTIFIC STATUS SUMMARY, 53(11), 67-72.

Sanders, M. E., Gibson, G., & Gill, H. S. (2007). Probiotics : Their Potential to Impact Human Health. CAST, (36), 1-4.

Selim, A. S., & Haider, G. (2014). Studies on the viabile bacteria of commercial probiotic products available in Bangladesh. World Journal of Microbiology, 1(2), 10-11.

Siddiqee, M. H., Sarker, H., & Shurovi, K. M. (2012). Assessment of probiotic application of lactic acid bacteria (LAB) isolated from different food items. Stamford Journal of Microbiology, 2(1), 10-11.

Suskovic, J., Kos, B., Beganovic, J., Pavunc, A. L., Habjanic, K., & Matosic, S. (2010). Antimicrobial activity - The most important property of probiotic and starter lactic acid bacteria. Food Technology and Biotechnology, 48(3), 296-301.

Vijarakumar, P. P., & Muriana, P. M. (2015). A Microplate Growth Inhibition Assay for Screening Bacteriocins against Listeria monocytogenes to Differentiate Their Mode-of-Action. Biomolecules, 5(2), 1179-1183.

Yusuf, M. A., & Abdul, T. H. (2013). Lactic Acid Bacteria : Bacteriocin Producer : A Mini Review.IOSR Journal Of Pharmacy, 3(4), 44-46.

Appendix 1

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
Sorbitan mono-oleate	1.0 ml
Di-potassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Tri-ammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Magnesium sulphate 4 H_2O	0.05
Agar	10.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Starch Agar

Component	Amount (g/ L)
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Methyl red Voges- Proskauer (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Gelatin Broth

Component	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
Final pH	6.8 ± 0.2 at 25°C

Sugar Fermentation Broth

Component	Amount (g/L)
Sugar	5.0
Trypticase	10.0
Sodium chloride	5.0
Phenol red	A very small amount until the broth turns red

T1N1

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

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Blood Agar Base

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

Skim Milk Agar

Component	Amount (g/L)
Skim milk powder	28.0
Casein enzymichydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
Casein enzymichydrolysate	5.0

Mueller-Hinton Agar (Himedia)

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

Lactobacillus MRS Broth (Himedia)

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 2

Reagents

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temeperature.

Malachite green (100 ml)

To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution was transferred to the reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was stored at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

Catalase Reagent (20 ml)

35 % hydrogen peroxide

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Nitrate Reagent A (100 ml)

5N acetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N,N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N) and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)[a] to form a colourless solution and stored at 4°C.

Ethyl Alcohol (95%)

95 ml of ethyl alcohol (100%) was added to 5 ml of distilled water. This solution was stored at room temperature.

Appendix 3

Instruments

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