Screening of Lactic Acid Bacteria with Antimicrobial Property Isolated from Fermented Foods

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

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Dedicated to

My Beloved Parents

DECLARATION

This is to declare that the research work exemplifying the results reported in this thesis paper entitled **"Screening of Lactic acid bacteria with antimicrobial property isolated from fermented foods"** submitted by Marium Momin, has been carried out under supervision of Professor Dr. M. Mahboob Hossain, Coordinator, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Bachelors of Science in Biotechnology, BRAC University, Dhaka and has not been submitted anywhere else for a degree or diploma.

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ABSTRACT

The purpose of this study was to isolate and biochemical identification lactic acid bacteria (LAB) with antimicrobial properties from fermented vegetables and dairy products. Twenty LAB isolates were separated from all the isolates which showed bactericidal effect when the agar well diffusion method was carried out to find the antimicrobial activity of the LAB isolates against seventeen (17) test organisms. According to the results of primary screening, the CFS (cell-free supernatants) of the LAB isolates showed antimicrobial effects against the indicator strains in this present work. Out of 20 LAB isolates, 12 were found to successfully inhibit *EPEC* (atypical) and *EPEC* (typical) respectively, 11 showed significant effect against *Enterobacter cloace*, 10 against *EAEC,* 9 worked against *Klebshiella spp., Salmonella typhi* & *Proteus vulgaris, 8 worked against Shigella flexneri, Streptococcus pyogens, Bacillus subtilis,* 7 worked against *ETEC* & *Shigella dysentry,* 6 were found to inhibit *Escherichia coli,* 4 worked against *Pseudomonas areuginosa* & *Streptococcus agalactiae,* 3 worked against *Streptococcus pneumoniae,* 2 showed inhibition against *Bacillus cereus.* LAB are known to have many health benefits and are mostly used as probiotics. As they can cause inhibition of food pathogens by the reduction of pH due to lactic acid production, hydrogen peroxide production and production of antimicrobial compounds such as bacteriocin. Bacteriocin is a high molecular weight protein. This study suggests that specific vegetables and dairy products contain abundant LAB species and nutrients which could be implemented as probiotic food products, living drugs and as an alternate to antibiotics.

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List of Abbreviations

LAB: Lactic Acid Bacteria

GRAS: Generally Recognized as Safe

MRS: de Man, Rogosa and Sharpe

MH: Mueller Hinton

ZOI: Zone Of Inhibition

MIU: Motility Indole Urea

TSI: Triple Sugar Iron

ºC: Degree Celsius

CFS: Cell free supernatant

GI: Gastrointestinal tract

et al: and others

spp.: Species

kDa: Kilo Dalton

STGG: Skim Milk-Tryptone-Glucose-Glycerol

ml: Milliliter

µl: Microliter

LPS: lipopolysaccharides

Conc.: Concentration

H_2O_2: Hydrogen peroxide

NADH: Nicotinamide Adenine dehydrogenase

BPB: Bacteriocin Producing Bacteria

Chapter 1

Introduction and Literature Review

1. Introduction

1.1 Background

It is well known that many lactic acid bacteria (LAB) are capable of producing a variety of antimicrobial compounds, which may contribute to their colonization of habitats and their competitive advantage over other bacteria (Garriga*et al.*, 1993). Besides production of lactic acid, this causes a drop in pH enough to inhibit the growth of certain strains, as its non-dissociated form triggers a lowering of the internal pH in sensitive bacteria that causes a collapse in the electrochemical proton gradient resulting in a bacteriostatic or bactericidal effect (O'Keffe*et al.*, 1999). LAB can produce other organic acids, di-acetyl, hydrogen peroxide, and bacteriocins (Nes & Johnborg, 2004).

Antimicrobial peptides (AMPs) are ubiquitous and natural antibiotics generated by a diverse range of microorganisms, plants, insect and mammalian cells. Recent attention has been drawn to AMPs as new antimicrobials to combat harmful microbes especially those resistant to conventional antibiotics. In the search for new antimicrobial agents, these may be used as templates for the design of novel drugs (Fedders, 2010); (Oyston, 2009). AMPs are divided into different groups based on their variable structural characteristics. Those AMPs produced by bacteria to kill or inhibit other bacteria are known as bacteriocins (Cleveland, 2001); (Sang, 2008).

Several bacteriocin-producing LAB strains have been isolated from milk and dairy products and particularly from goat milk (Cocolin*et al.*, 2007). It is generally accepted that bacteriocins produced by LAB are more active against Gram positive bacteria, however KSA2386, ST34BR and lacticin NK24 are bacteriocins produced by *L. lactis* that present a larger spectrum of activity, being active against Gram-negative bacteria as well (Todorov & Dicks, 2004); (Pirad, 1994).The fermentation of vegetables is due primarily to the activity of naturally occurring lactic acid bacteria. Fermentation is an economic means of preservation and bulk storage of produce and the production of unique flavor and other quality characteristics (I. M. Pe´rez-Dı´az, 2013).

1.2 Literature Review

1.2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are important in foods as potential probiotics and also due to the ability to produce antimicrobial compounds. LAB are present in fermented food products and help to improve shelf life and enhance the flavor of the food. They also produce metabolites such as bacteriocins to prevent the growth of undesirable or pathogenic bacteria. (Goh & Philip, 2015). The production of bacteriocins by some LAB also contributes to the inhibition of spoilage bacteria, thereby extending the shelf life of the food (Deegan*et al.*, 2006).

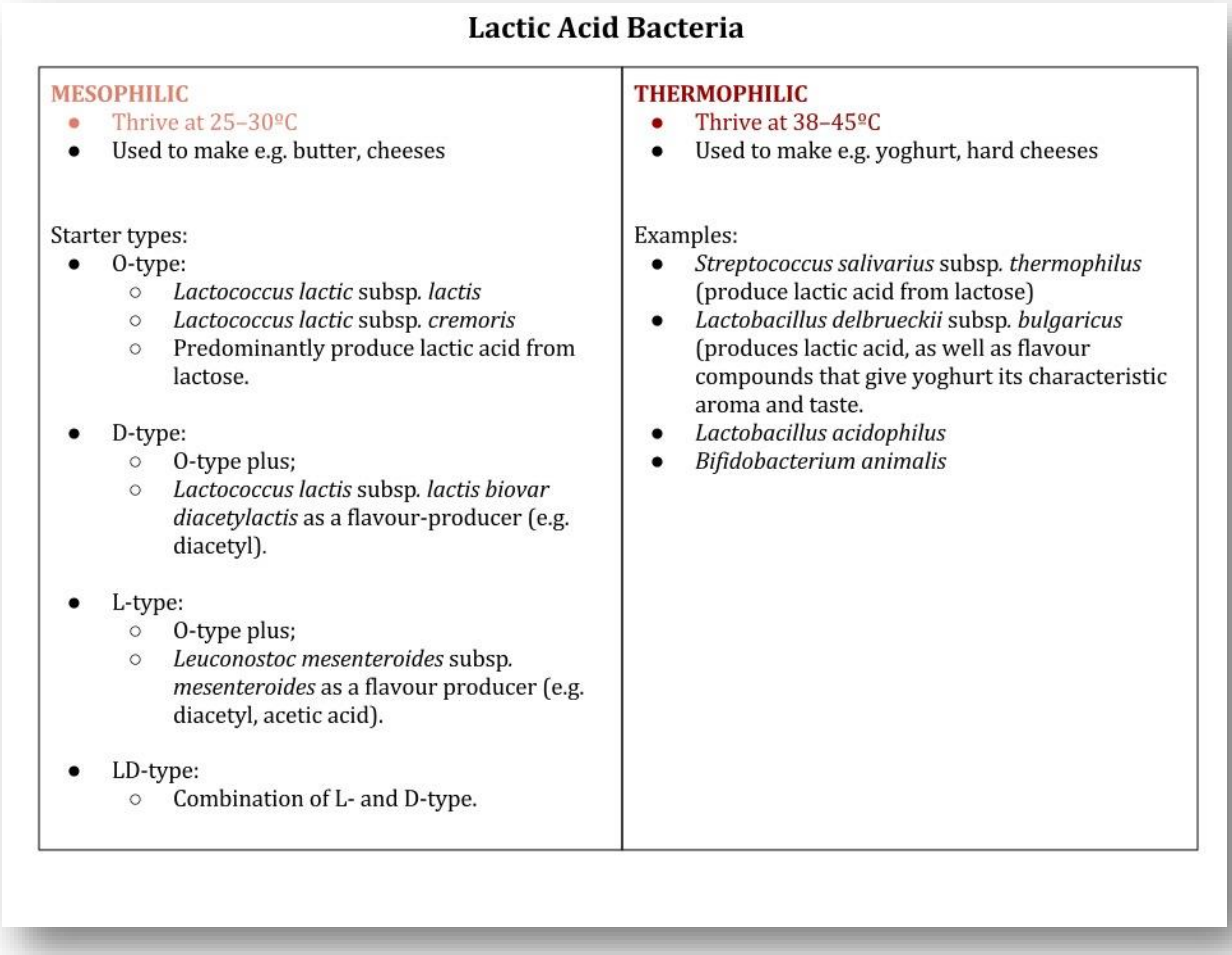


Figure 1.1: Ubiquitous properties of LAB

Lactic acid bacteria (LAB) are characterized as Gram-positive cocci or rods, non-aerobic but aerotolerant, able to ferment carbohydrates for energy and lactic acid production. The metabolic pathway from glucose may be homofermentative or heterofermentative. In the first case two molecules of lactate are generated (as in *Streptococcus* and *Lactococcus*), and in the second, lactate, ethanol and carbon dioxide are produced, as in *Leuconostoc* and some lactobacilli (Par Caron *et al.*, 2007).

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 & Hill, 2001).

Table 1.1: Key genera species of microbes studied and used as probiotics (Bonner, 2007)

No.	Genus	Species
1.	*Lactobacillus*	▪ *acidophilus* ▪ *brevis* ▪ *fermentum* ▪ *plantarum* ▪ *reuteri*
2.	*Bifidobacterium*	▪ *adolescentis* ▪ *bifidum* ▪ *infantis* ▪ *longum*
3.	*Streptococcus*	▪ *thermophilus* ▪ *salivarius*
4.	*Enterococcus*	▪ *faecium*

Moreover, *Lactobacillus acidophilus, L. plantarum, L. casei, L. caseirhamnosus, L. delbrueckiibulgaricus, L. fermentum, L. reuteri, Lactococcuslactislactis, Lactococcuslactiscremoris, Bifidobacteriumbifidum, B. infantis, B. adolecentis, B. longum, B. breve, Enterococcus faecalis, Enterococcus faecium,* are some of the most common species (Garrity, 1984), and some strains are recognized as probiotics (Parada *et al.*, 2003). Lactic acid bacteria are also able to produce small organic substances that contribute with aroma and give specific organoleptic attributes to the products (Caplice & Fitzgerald, 1999).

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. They have also been used as flavor and texture producers (Carr & Hill, 2001).

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS) microorganisms and have been used in food fermentation for a long time. LAB are usually known as safe, and have an important role in the preservation of foods and fermented products. They can be used as natural competitive microbiota or as specific starter cultures under controlled conditions. Some of these bacteria produce antagonistic substances, called bacteriocins, which in small amounts are very active against pathogens (Koponen, 2004). A balance among microbial groups present in human gut is crucial for maintaining health. When this balance is disturbed, the host–microbe relationship can progress towards a disease state. Thus, the maintenance of micro biota equilibrium is important to preserve and to promote health. Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called “probiotics” in the intestinal tract (Nigam *et al.*, 2012).Sugar fermentation followed by a reduction in pH due to the production of lactic and other organic acids is an important factor for the inhibition of growth of undesired microorganisms. The low pH makes organic acids liposoluble, allowing them to break through the cell membrane and reach the cytoplasm of pathogens (Haller *et al.*, 2001).The competition for essential nutrients, accumulation of D-amino-acids and diminution of the oxido-reductive potential also contribute to their inhibitory effect.

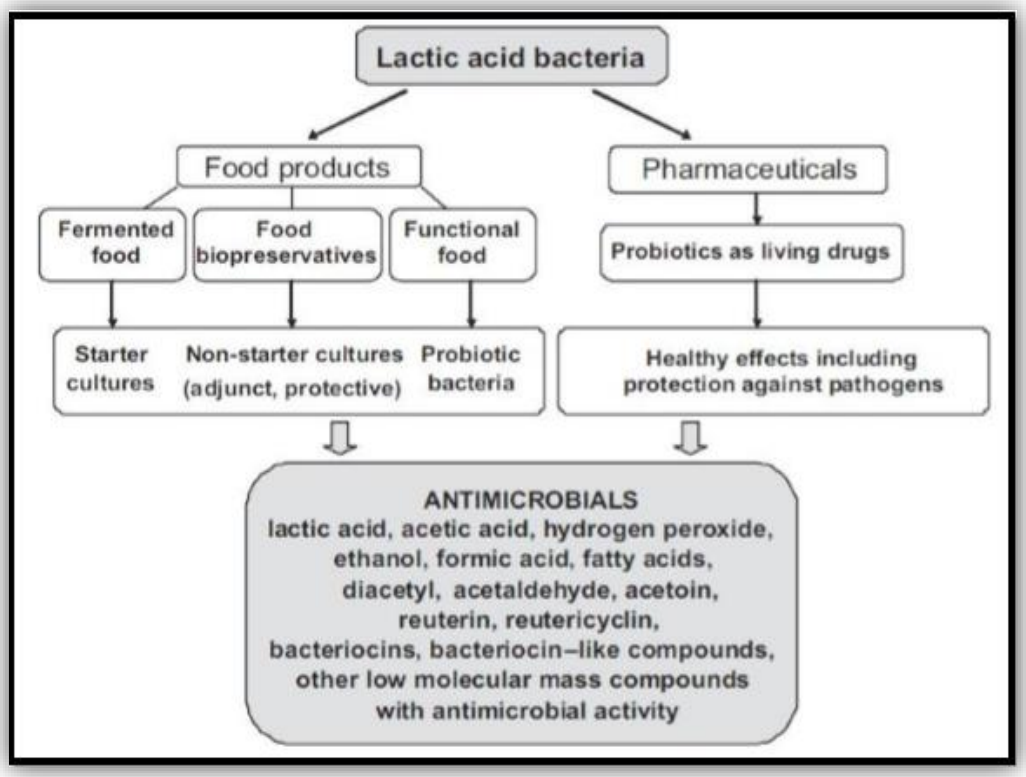


Figure 1.2: The various sectors that LAB can contribute (Suskovic, 2010)

1.2.2 Antimicrobial Property by Lactic Acid Production

Lactic acid produced by lactic acid starter culture bacteria functions as a natural antimicrobial component, having a generally recognized as safe status. Lactic acid is able to inhibit the growth of many types of food spoilage bacteria, including gram-negative species of the families Enterobacteriaceae and Pseudomonadaceae (Doores, 1993). Among other organic acids, lactic acid is recognized as a bio-preservative in naturally fermented products. The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane of organisms, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force (Ray & Sandine, 1992).

Table 1.2: Metabolic Products and mode of Antagonistic action of LAB (Suskovic, 2010)

Metabolic Product	Mode of Antagonistic Action
Carbon Dioxide	Inhibits decarboxylation Reduces membrane permeability
Diacetyl	Interacts with arginine-binding proteins
Hydrogen peroxide/ Lactoperoxidase	Oxidizes basic proteins
Lactic Acid	Undisssociated lactic acid penetrates the membranes, lowering the intracellular pH. Interferes with metabolic processes such as oxidative phosphorylation.
Bacterocins	Affects membranes, membrane-associated replication and DNA/protein synthesis.

1.2.3 Probiotics

There are a large number of probiotic foods which date back to ancient times which are mostly originated from fermented foods as well as cultured milk products (Goh & Philip, 2015). The quest to find food ingredients with valuable bioactive properties has encouraged interest in LAB with probiotic attributes such as antibacterial activity against pathogenic microorganisms, antiviral activity, anti-yeast property anti-mutagenic, anti-platelet aggregation, and anti-oxidant attributes etc. In general, it is believed that probiotics help keep up the balance between harmful and beneficial bacteria in the gut thus maintaining a healthy digestive system (Salminen & Wright, 2011). The health claims of probiotics range from regulation of bowel activity and well-being to more specific actions such as, antagonistic effect on the gastro enteric pathogens like *Clostridium difficile*, *Campylobacter jejuni*, *Helicobacter pylori* and Rotaviruses and

others (Gopal*et al.*, 2001). Some are known to neutralize food mutagens produced in colon, shifting the immune response towards a Th2 response, alleviating allergic reactions, and lowering serum cholesterol. The mechanism of action of probiotics with antibacterial properties is maybe due to the production of bacteriocins such as nicin (Doron & G.S.L., 2006) or lowering the pH by producing acidic compounds like lactic acid (Tannock, 2005).

Probiotic strains compete with other infectious bacteria for nutrients and cell-surface and help toward them off by inhibiting their colonization. A few strains are also known to produce active enzymes which inhibit other pathogenic bacteria. The health benefits of probiotics have always been investigated with regard to their capability to sustain their availability, viability (Piard & D.M., 1991), digestibility, and rendering of their health benefits to the host without altering the safety and the organoleptic properties of the food in which they have been incorporated. Today, viable probiotic strains with beneficial functional properties are available in the market as components of foods and beverages, in fermented dairy products like yogurt (Goktepe*et al.*, 2006) or as probiotic fortified foods as well as food preservatives.

Lactobacillus acidophilus, L. plantarum, L. casei, L. caseirhamnosus, L. delbrueckiibulgaricus, L. fermentum, L. reuteri, Lactococcuslactislactis, Lactococcuslactiscremoris, Bifidobacteriumbifidum, B. infantis, B. adolecentis, B. longum, B. breve, Enterococcus faecalis, Enterococcus faecium, are some of the most common species (Garrity, 1984), and some strains are recognized as probiotics (Parada *et al.*, 2003).

Probiotics are living microorganisms that are taken for their preventive or therapeutic effects on a wide variety of disease states, many of which are common in the pediatric population. A wide variety of probiotic species are available, but Lactobacillus species are the most investigated. The majority of probiotic use revolves around gastrointestinal diseases. Probiotics are active living microorganisms that have a defined health benefit when ingested in sufficient quantity. These microorganisms are usually consumed with the intent to replace or increase an individual's micro-flora. Probiotics must be resistant

to stomach acid and bile to survive passage through the digestive tract, must be able to proliferate and colonize the gut, and must be able to attach and adhere to the lining of the GI tract (Heiberger *et al.*, 2014).

1.2.3.1 Effects of Probiotics

A wide variety of probiotic sp.are available, but the most investigated are species of *Lactobacillus* and *Bifidobacterium* (Didari*et al.*, 2014), (Marchand *et al.*, 2012).

Table 1.3: Variety of probiotic spp. available as different products (Heiberger*et al.*, 2014)

STRAIN	BENEFITS	PRODUCTS
Bifidobacterium animalis DN-173 010 (Bifidis regularis)	Gut health and faster digestion	Dannon Activia yogurt
Bifidobacterium infantis 35624 (Bifantis)	Digestive health; Alleviates symptoms of irritable bowel syndrome	Procter & Gamble's Align dietary supplement
Bifidobacterium lactis Bb-12	Helps immune system and digestive health	Yoplait Yoplus yogurt
Lactobacillus casei DN-114 001 (*L. casei Immunitas*)	Helps immune system; lessens duration of colds and flus in older people; eases diarrhea in children and people taking antibiotics	Dannon DanActive dairy drink
Lactobacillus casei Shirota	Helps immune system and digestive health	Yakult fermented dairy drink
Lactobacillus rhamnosus GG	Digestive health, infant diarrhea	Culturelle dietary supplement
Lactobacillus rhamnosus GR-1 in combination with *Lactobacillus reuteri*	Improved vaginal health; helps eradicate vaginal infections	RepHresh Pro-B and Fem-Dophilus, both dietary supplements
Lactobacillus reuteri DSM 17938	Eases infant colic; helps immune system; lessons antibiotic-associated diarrhea. When blended with another strain, helps treat bleeding gums	BioGaia chewable tablets, drops, and lozenges
Saccharomyces boulardii yeast	Helps prevent and treat antibiotic-associated diarrhea	Florastor dietary supplement

Probiotics induce an increase in the number of intestinal bacteria. Beneficial effects are likely due to a multitude of actions, including modulation of immune function leading to stimulated host defense mechanisms; down regulation of inflammatory cytokines resulting in decreased chronic gut inflammation (Vieira *et al.*, 2013).

1.2.4 Bacteriocin

Bacteriocins have antimicrobial activities against food-spoiling bacteria and food-borne pathogens (Wang *et al.*, 2016). Bacteriocins are antimicrobial peptides which are ribosomally-synthesized of bacterial origin that are active against a variable spectrum (from narrow to broad) of bacteria. These peptides play a major role to control non-indigenous or pathogenic organisms (Dover *et al.*, 2008). Production of these antimicrobial peptides plays an important role in bacterial competition, allowing survival advantages to the producing strain (Ruiz *et al.*, 1994). 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. 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 & Abdul, 2013).These ribosomally synthesized proteinaceous compounds are bactericidal only toward Gram-positive bacteria, which can be explained by the additional protective layer of Gram-negative composed of phospholipids, proteins and LPS (Dortu, 2009), (Abee*et al.*, 1995).Moreover, bacteriocins are innocuous due to proteolytic degradation in the gastrointestinal tract (Cintas *et al.*, 1995). Bacteriocins from Gram-positive bacteria are nowadays classified into two major classes (Cotter *et al.*, 2005):

- **i.** The lantibiotics (class I; bacteriocins containing post-transnationally modified residues)
- **ii.** The non-lantibiotics (class II; bacteriocins with non-modified residues except for the formation of disulfide bridges and circular bacteriocins).

Class II is divided into four subgroups:

- Class II a (pediocin-like bacteriocins)
- Class II b (two-peptide bacteriocins)
- Class II c (cyclic bacteriocins)
- Class II d (linear non-pediocin-like one-peptide bacteriocins)

Bacteriocin may have a narrower spectrum against other bacteria than antibiotics. Several types of bacteriocins can be found in several bacterial species.

Table 1.4: Several types of bacteriocins with several bacterial spp. (Cotter, 2005)

Bacteriocin	Bacterial Species
Acidolin	*Lactobacillus acidophiluus*
Adidophilin	*Lactobacillus acidophiluus*
Lactacin B	*Lactobacillus acidophiluus*
Lactacin F	*Lactobacillus acidophiluus*
Bulgarin	*Lactobacillus bulgaricas*
Plantaracin SIK-83	*Lactobacillus plantarum*
Plantaracin A	*Lactobacillus plantarum*
Lactolin 27	*Lactobacillus helveticus*
Helvetecin J	*Lactobacillus helveticus*
Reuterin	*Lactobacillus reuteri*
Lactobacillin	*Lactobacillus brevis*

It is generally accepted that bacteriocins exert their inhibitory action by formation of pores in the cytoplasmic membrane of Gram-positive bacteria. These cells differ in their sensitivity mainly because of difference in membrane composition and fluidity. Self-evidently, bacteriocin producers exhibit specific immunity against their bacteriocin. This is accomplished by the production of dedicated immunity (Lucke, 2000). 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 & Abdul, 2013).

Bacteriocins that have often been mooted as potentially food-grade to improve food safety can reduce the prevalence of foodborne diseases and also help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties (Martinez *et al.*, 2003).

This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources (Deegana*et al.*, 2006). 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 (Yusuf & Hamid, 2013). 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.

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activitiy and spectra of action (Saavedra *et al.*, 2004).

1.3. Objectives

The aim of the present work was to isolate LAB with antimicrobial properties, identification of the antimicrobial agents (preferably bacteriocin) responsible for the aforementioned antagonistic effect.

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 (Siddiqee *et al.*, 2012). As there is an increase in the emergence of new pathogens and antibiotic resistant pathogens, the purpose of this study is to find new and more antimicrobial compounds among the local species of LAB. Comparing the well diffusion assay and spot-on-lawn method and to determine the most reliable method for detection of anti-microbial activity against other pathogenic bacteria and also to find new strains of Lactic Acid Bacteria that produce anti-microbial compounds.

Moreover, there are very few published works in Bangladesh regarding the effectiveness of LABs against pathogens (Siddiqee *et al.*, 2012). For this reason performing the study could hovel some ray on that, through the assessment of the antimicrobial activity of whole cells LAB and extracellular material against certain pathogens.

Chapter 2

Materials and Methods

2. Materials and methods

2.1 Work place

This research work was carried out in the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Dhaka 1212, Bangladesh.

2.2 Collection and processing of Samples

Lactic Acid Bacteria were collected from fresh vegetables and dairy products. There were eleven food samples from where 2-3 different LAB isolates were found on the MRS agar plate after 48 hours incubation at 30°C.

Table 2.2: Sources of LAB isolates

No.	**Sources**	**Types of sample**
01.	MatriVandar Sweet Curd	yoghurt
02.	Arong Sour (Light yellowish colony)	yoghurt
03.	Arong Sour (white colony)	yoghurt
04.	Shakti Sweet (white colony)	yoghurt
05.	Shakti Sweet (Light yellowishcolony)	yoghurt
06.	Delicia sweet curd	yoghurt
07.	Buttermilk (white colony)	dairy product
08.	Buttermilk (off-white colony)	dairy product
09.	Dhaka Cheese	dairy product
10.	Cauliflower(white colony)	vegetable
11.	Cauliflower(light yellowishcolony)	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(offwhite colony)	vegetable
19.	Cabbage(light yellowish colony)	vegetable
20.	Cabbage(offwhite colony)	vegetable

2.2.1 Growth of bacteria from food samples

In case of solid samples, growth of LAB was gained through fermentation. Fermentation was done by cleaning and dicing the samples before placing them in distilled water. Fermented samples (1 ml) were directly inoculated into MRS (de Man, Rogosa, Sharpe) broth and incubated for 48 hours at 30° C.

- The solid samples were washed thoroughly under distilled water, after being diced. Then they were placed into volumetric flasks containing distilled water. The volumetric flasks were covered tightly with aluminum foil and para-film tape, to create an environment that is favorable for fermentation.
- The sorted samples were left for three or four weeks, in room temperature.
- Afterwards, 1 ml of this solution was placed into MRS (de Man, Rogosa and Sharpe) agar plate from the flasks containing samples using a pipette and incubated for 48 hours at 30° C. Firstly a serial dilution of x2 was prepared using 0.9% saline solution. 200µl of this was dropped onto a MRS agar plate, and then the spread plate technique was applied. Distinct colonies were expected after 48-72 hours of anaerobic incubation at 30°C.
- The incubated plates were taken to subculture the obtained different colonies using streaking technique to ensure that each new MRS plates contain single type pure cultures.
- After that, from every pure culture plates single or two colonies were taken for inoculation into MRS broth.
- The inoculated MRS broths were vortexed and then incubated for 48 hours (until turbid) anaerobically at 30° C. After this, cultures from the plates and broths were preserved for antimicrobial assay.
- Catalase test, Gram stain and endospore stain were done at first to reassure the isolates as LAB.

2.2.2 Primary Detection of LAB

Determination of LAB was primarily done based on microscopic observations and catalase test.

1. **Gram staining:** All LAB isolates were gram positive; therefore the gram staining method was conducted. Gram staining was done using standard procedure (Cappuccino & Sherman, 2005).

2. **Catalase Test:** This test was done first on all the samples to separate the possible LAB species (catalase negative) from the non-LABs (catalase positive) (Cappuccino & Sherman, 2005).
   - 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.

2.2.3 Storage and Subculture of LAB isolates

After the microscopic evaluations and the two other biochemical tests, the isolates matched with the characteristics and those were kept in the refrigerator at 4°C for further experiments. MRS media is a synthetic complex, according to previous studies (Abas *et al.*, 2012). The collected isolates were streaked onto MRS agar plates in every two weeks and stored at 4°C. The four-way streak plate inoculation was followed for this experiment (Cappuccino & Sherman, 2005).

2.2.4 Long term storage

Each of the isolates was preserved in T_1N_1 agar, at room temperature and also in Skim Milk-Tryptone-Glucose-Glycerol (STGG) media at -20°C.

2.3 Screening of LAB isolates for antimicrobial activity:

The agar well diffusion method is the best suitable to test for the antimicrobial property of LAB. This method requires a 24 hours culture of the pathogens which was suspended in saline before performing lawn culture, which is used as an indicator strain by spreading the cell suspension over the surface of Muller Hinton 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 CFS (cell free supernatant) obtained from the MRS broth containing LAB isolates. Antimicrobial activity of the CFS was observed after the 24 hours incubation at 37°C. Zone Of Inhibition around the wells indicates antimicrobial activity and the results were considered positive if the diameter (milimeter) of the ZOI was greater than ten millimeter (Kazemipoor *et al.*, 2012).

2.3.1 Well diffusion technique

i. Ten milliliter MRS broths were heavily inoculated with sample isolates. The broths were vortexed and anaerobically incubated at 30°C for 48 hours.
ii. The MRS broth cultures were centrifuged at ten thousand rpm for 15 minutes.
iii. The supernatants were transferred into a sterile micro centrifuge tube or micro-centrifuge tube gently. The supernatant should not be mixed with the pellet.
iv. The saline solutions were used to prepare lawns on MH agar plates. For each indicator strain a sterile cotton swab was used.
v. Afterwards, with a sterile cork-borer, 5mm wells were punctured into the MH agar plate (Nowroozi *et al.*, 2004). The punctured gel was removed with the help of a sterile inoculating needle.
vi. Using a micropipette 50µl (Lavanya *et al.*, 2013) of each sample's CFS was added to a well. It is important to fill up the wells within 15 minutes of spread-plating with the indicator strains.
vii. The MH agar plates were placed in the incubator for 24 hours at 37°C.
viii. Observation for any ZOI that is 1mm or greater was conducted.

2.4 Identification of LAB isolates

Screening the LAB for antimicrobial activity was determined based on two following characteristics:

i. Morphological tests (Colony morphology was observed under a compound microscope at 100X.)
ii. Biochemical tests

1) Growth at different temperature

- Streaking of organisms was performed onto MRS plates.
- These plates were incubated at 15ºC and 45 ºC for 48 hours.

2) Spore stain

Using a pure colony of the *Lactobacillus* control, a spore stain was conducted. The spore stain is used to determine whether the bacteria produce spores or not.

- When stained with malachite green, then counter stained with safranin, spores will turn a bright green, whereas vegetative cells will stain a dark pink.
- Since *Lactobacillus* is a non-spore forming bacteria then a positive result for *Lactobacillus* would be dark pink stained cells.

3) Oxidase test

- A Whatman filter paper (1mm) was soaked with the oxidase reagent.
- One loop which contains a colony from pure culture bacteria was streaked on it.
- Within 30 seconds to 1 minute, if purple color appears over the bacteria in the paper is a positive result; if no colour appears then it will be conducted as a negative result.
- Delayed reactions are ignored and concluded as negative result.

4) 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 two-third way of the medium in the test tube approximately at the center.
- 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.

5) Nitrate reduction test

- Bacteria was inoculated with the help of a loop in the nitrate broth and incubated for 24 hours.
- Nitrate reduction test reagents A and B were added after incubation period.
- The observation of changes in the color from a light pink to a deep red within a few moments by the addition of reagents A and B signified that nitrate reductase enzyme is present.
- After the addition of zinc powder; if no colour change was observed then it was considered as a positive result and confirmed the presence of both nitrate reductase and nitrite reductase. If red colour appeared after this step then it can confirm a negative result for nitrate reduction.

6) Casein hydrolysis test

- Five hundred milliliter distilled water was collected and divided into two conical flasks.
- Both the conical flasks, one is dissolved with nutrient agar and the other with distilled water, was autoclaved for 15 minutes.
- Skim milk agar was mixed into the autoclaved distilled water once it had been slightly cooled.
- The two solutions were mixed together and no lumps should not present. Then, the solution was poured into sterile petri dishes.
- The isolates were inoculated on the agar. Then they were incubated at 37ºC for 24 hours. If any clear areas are observed around the growth of the organism then the result was confirmed as a positive result.

Clear zones: Clear zones around the growth of the organisms indicate positive result of casein hydrolysis test.

No clear zones: If the agar appears opaque around the growth of the organism, then it means the organism does not hydrolyze casein.

7) Gelatin hydrolysis test

- Mediapreparation: Five gram peptone, three gram beef extract and one hundred and twenty gram liquid gelatin per liter were added together in the distilled water.
- Two milliliter of the medium was dispensed into vials, and then autoclaved.
- The media was inoculated with an inoculating needleand then incubated at 37ºC for 48 hours.
- The cultures were placed in the refrigerator at 4ºC for 30 minutes and then 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.

8) Triple Sugar Iron Test

- Using an inoculating needle, organism was taken up and stabbed into slanted TSI agar till to its butt and then withdrawn and streaked over the slant surface. The lid was loosely closed to ensure the passing of air.
- The TSI agar was incubated at 37ºC for 24 hours.

9) Carbohydrate fermentation tests:

Fermentation of maltose, rhamnose, tetrahalose, xylose, mannitol, galactose, glucose, lactose, starch and sucrose were done.

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

Chapter 3

Results

3.1. Growth of LAB isolates on selective Agar

Twenty samples tested and showed the maximum growth on MRS media. After the inoculation, bacteria took 48 hours to show the maximum growth. Colonies from the plates were opaque; rounds shaped but have different colours and morphology in each of the petri dish.

Figure 3.1: Bacterial growth on MRS Agar plates (a and b)

3.2. Gram staining results

Gram staining was done on colonies to differentiate the LAB isolates from the non-LAB isolates. Morphology was observed under a compound microscope at 100X.

Table 3.2: Gram staining test results

No.	Source and colony colour of the isolates	Gram Positive/ Gram Negative	Morphology
1.	MatriVandar Sweet Curd	Gram positive	cocci
2.	Arong Sour (Light yellowish colony)	Gram positive	rod
3.	Arong Sour (white colony)	Gram positive	rod
4.	Shakti Sweet (white colony)	Gram positive	rod
5.	Shakti Sweet (light yellowish colony)	Gram positive	rod
6.	Turnip (white colony)	Gram positive	rod
7.	Turnip (light yellowish colony)	Gram positive	rod
8.	Carrot (white colony)	Gram positive	rod
9.	Carrot (light yellowish colony)	Gram negative	rod
10.	Brinjal (off-white colony)	Gram positive	rod
11.	Brinjal (white colony)	Gram positive	cocci
12.	Buttermilk (off-white colony)	Gram positive	rod
13.	Buttermilk (white colony)	Gram positive	rod
14.	Cabbage (white colony)	Gram positive	rod
15.	Cabbage (off-white colony)	Gram positive	rod
16.	Cabbage (light yellowish colony)	Gram positive	rod
17.	Cauliflower (white colony)	Gram positive	rod
18.	Cauliflower (light yellowish colony)	Gram positive	rod
19.	Delicia Sweet Curd	Gram positive	cocci
20.	Dhaka Cheese	Gram positive	rod

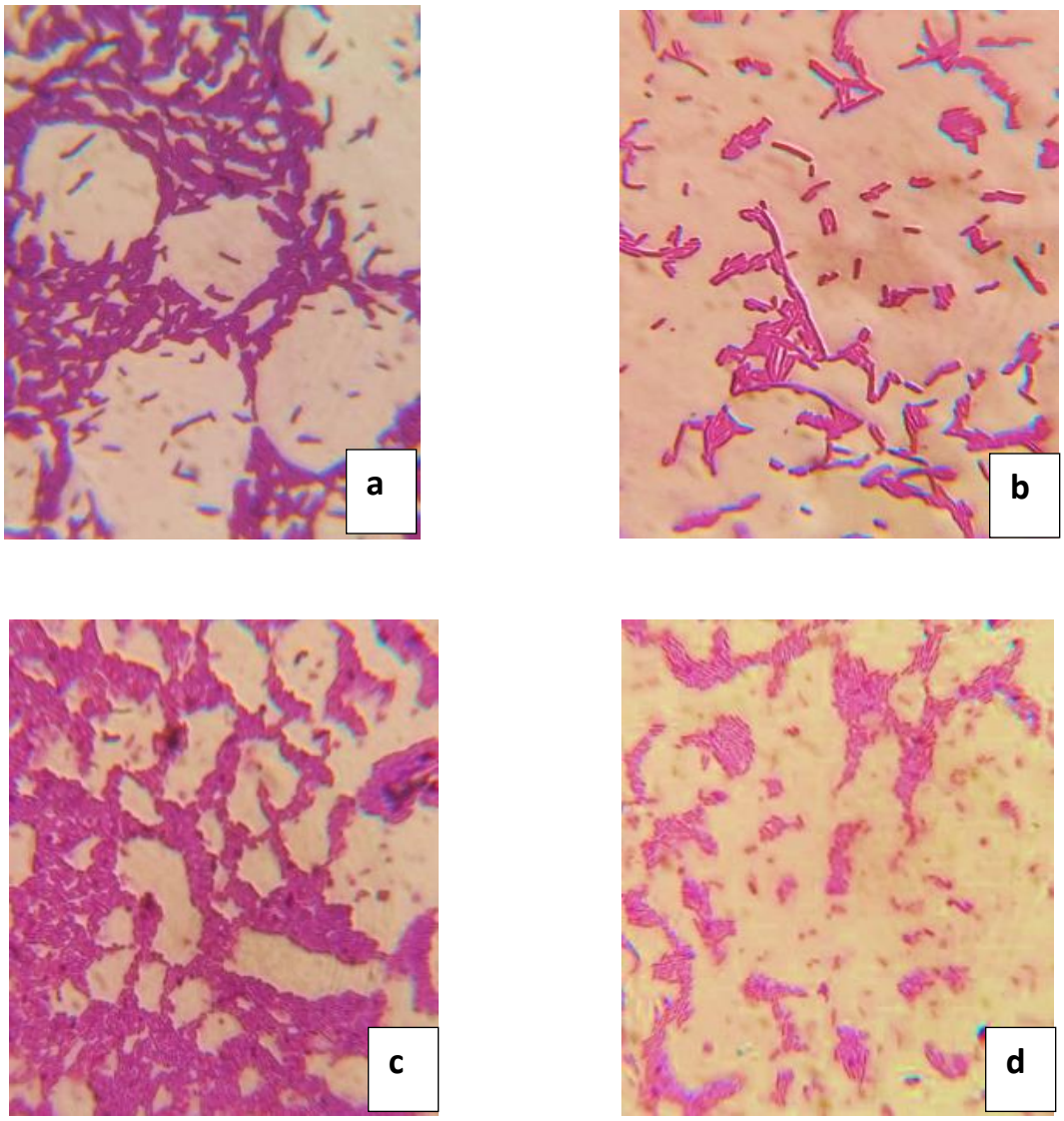


Figure 3.2: Microscopic observations of the isolates after gram staining.
a. *Lactobacillus plantarum* **b.** *Lactobacillus composti* **c.** *Lactobacillus saniviri*
d. *Lactobacillus salivarius*

3.3 Catalase Test (35% Hydrogen Peroxide)

Some of the LAB isolates shown to be catalase negative and some shown to be catalase positive. Matrivandar sweet curd, Buttermilk (white & off-white colony), Turnip (light yellowish colony), Delicia sweet curd, Carrot (light yellowish colony), Dhaka cheese isolates are catalase positive and others are catalase negative.

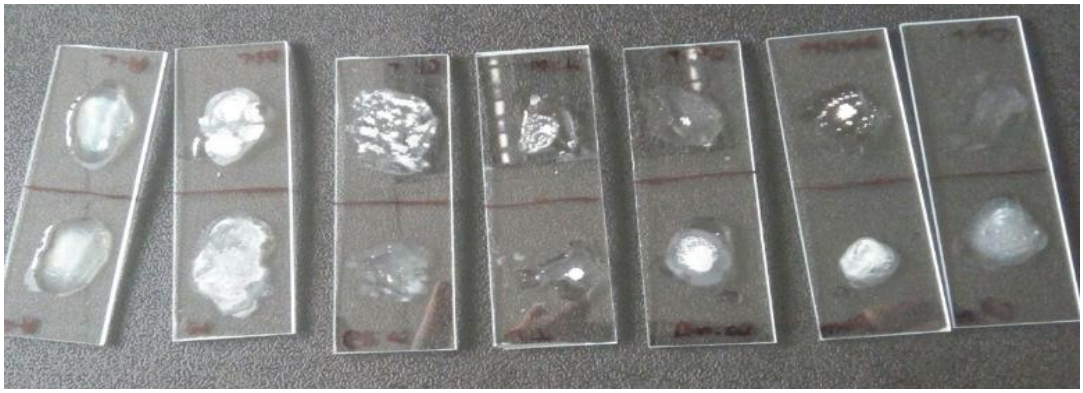
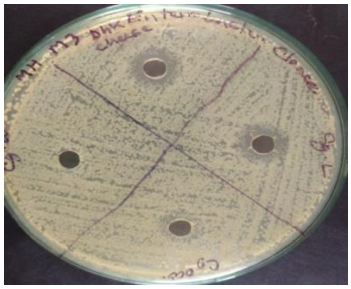


Figure 3.3: Catalase test

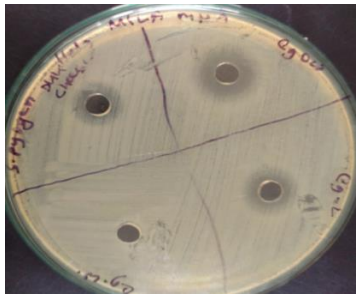
3.4 Primary screening results (the agar well diffusion method): Zone of inhibition (ZOI) observed from different samples on the MH agar using well diffusion method against seventeen tested organisms. Some of the results are showed by the figure below.

a. Six isolates against *Streptococcus pneumoniae*



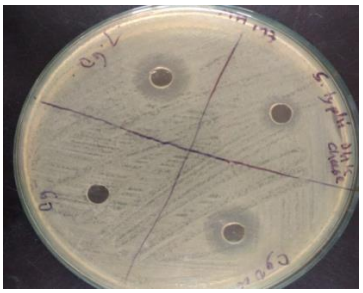
b. Four isolates against *Enterobacter cloacae*

c. Four isolates against *Klebshiella sp.*



d. Four isolates against *Streptococcus pyogens*

e. Four isolates against *Streptococcus agalactiae*



f. Four isolates against *Salmonella typhi*

Figure 3.4: ZOI by the LAB isolates against test organisms

Table 3.4: Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
1.	*EPEC* (typical)	Arong sour curd (white colony)	14
		Shakti sweet curd (white colony)	13
		Arong sour curd (light yellowish colony)	15
		Shakti sweet curd (light yellowish colony)	14
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	11
		Delicia sweet curd	12
		Dhaka Cheese	11
		Cauliflower (white colony)	14
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	14
		Cabbage (offwhite colony)	17
		Cabbage (white colony)	13
2.	*EPEC* (atypical)	Arong sour curd (white colony)	14
		Shakti sweet curd (white colony)	12
		Arong sour curd (light yellowish colony)	16
		Shakti sweet curd (light yellowish colony)	13
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	12
		Delicia sweet curd	14
		Dhaka Cheese	14
		Cauliflower (white colony)	14
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	14
		Cabbage (offwhite colony)	17
		Cabbage (white colony)	13

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
3.	ETEC	Arong sour curd (white colony)	13
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	16
		Shakti sweet curd (light yellowish colony)	11
		Matrivandar sweet curd	11
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	11
		Delicia sweet curd	14
		Dhaka Cheese	16
		Cauliflower (white colony)	13
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	15
		Cabbage (offwhite colony)	16
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
4.	EAEC	Arong sour curd (white colony)	13
		Shakti sweet curd (white colony)	13
		Arong sour curd (light yellowish colony)	12
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	12
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	13
		Dhaka Cheese	14
		Cauliflower (white colony)	12
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	15
		Cabbage (offwhite colony)	16
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
5.	*Escherichia coli* ATCC2525922	Arong sour curd (white colony)	13
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	12
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	14
		Delicia sweet curd	no zone
		Dhaka Cheese	no zone
		Cauliflower (white colony)	13
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	11
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	no zone
		Cabbage (offwhite colony)	no zone
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
6.	*Shigellaflexneri*	Arong sour curd (white colony)	15
		Shakti sweet curd (white colony)	14
		Arong sour curd (light yellowish colony)	no zone
		Shakti sweet curd (light yellowish colony)	13
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	13
		Cauliflower (white colony)	16
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	11
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	14
		Cabbage (offwhite colony)	17
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
7.	*Shigelladysentry*	Arong sour curd (white colony)	11
		Shakti sweet curd (white colony)	11
		Arong sour curd (light yellowish colony)	13
		Shakti sweet curd (light yellowish colony)	12
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	no zone
		Cauliflower (white colony)	16
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	11
		Cabbage (offwhite colony)	14
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
8.	*Streptococcus pyogens*	Arong sour curd (white colony)	12
		Shakti sweet curd (white colony)	13
		Arong sour curd (light yellowish colony)	15
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	14
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	14
		Cauliflower (white colony)	16
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	15
		Cabbage (offwhite colony)	17
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (millimeter)
9.	*Streptococcus pneumoniae*	Arong sour curd (white colony)	no zone
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	no zone
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	no zone
		Cauliflower (white colony)	15
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	18
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	20
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	no zone
		Cabbage (offwhite colony)	no zone
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
10.	*Streptococcus agalactiae*	Arong sour curd (white colony)	15
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	no zone
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	14
		Cauliflower (white colony)	14
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	no zone
		Cabbage (offwhite colony)	18
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
11.	*Bacillus cereus*	Arong sour curd (white colony)	14
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	no zone
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	no zone
		Cauliflower (white colony)	17
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	no zone
		Cabbage (offwhite colony)	no zone
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
12.	*Bacillus subtilis*	Arong sour curd (white colony)	no zone
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	12
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	13
		Delicia sweet curd	no zone
		Dhaka Cheese	16
		Cauliflower (white colony)	15
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	11
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	21
		Cabbage (offwhite colony)	15
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
13.	*Pseudomonas areuginosa*	Arong sour curd (white colony)	no zone
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	no zone
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	16
		Cauliflower (white colony)	13
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	15
		Cabbage (offwhite colony)	15
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
14..	*Klebshiella* sp.	Arong sour curd (white colony)	13
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	13
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	13
		Delicia sweet curd	no zone
		Dhaka Cheese	14
		Cauliflower (white colony)	13
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	11
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	16
		Cabbage (offwhite colony)	18
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
15.	*Enterobacter cloacae*	Arong sour curd (white colony)	13
		Shakti sweet curd (white colony)	14
		Arong sour curd (light yellowish colony)	13
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	17
		Cauliflower (white colony)	15
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	13
		Turnip (light yellowish colony)	13
		Carrot (white colony)	15
		Carrot (light yellowish colony)	12
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	19
		Cabbage (offwhite colony)	15
		Cabbage (white colony)	no zone

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
16.	*Proteus vulgaris*	Arong sour curd (white colony)	no zone
		Shakti sweet curd (white colony)	no zone
		Arong sour curd (light yellowish colony)	12
		Shakti sweet curd (light yellowish colony)	no zone
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	no zone
		Delicia sweet curd	no zone
		Dhaka Cheese	14
		Cauliflower (white colony)	13
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	12
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	11
		Carrot (light yellowish colony)	13
		Brinjal (off-white colony)	12
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	13
		Cabbage (offwhite colony)	17
		Cabbage (white colony)	no zone

Table 3.4 (continued): Antimicrobial activity of CFS of the LAB isolates against test organisms

No.	Name of the tested organism	Source and colony colour of the isolates	Inhibition Zone (milimeter)
17.	*Salmonella typhi*	Arong sour curd (white colony)	18
		Shakti sweet curd (white colony)	15
		Arong sour curd (light yellowish colony)	18
		Shakti sweet curd (light yellowish colony)	14
		Matrivandar sweet curd	no zone
		Buttermilk(white colony)	no zone
		Buttermilk (off-white colony)	14
		Delicia sweet curd	no zone
		Dhaka Cheese	12
		Cauliflower (white colony)	13
		Cauliflower (light yellowish colony)	no zone
		Turnip (white colony)	no zone
		Turnip (light yellowish colony)	no zone
		Carrot (white colony)	no zone
		Carrot (light yellowish colony)	no zone
		Brinjal (off-white colony)	no zone
		Brinjal (white colony)	no zone
		Cabbage (Light yellowish colony)	21
		Cabbage (off-white colony)	17
		Cabbage (white colony)	no zone

3.5. Identification of organisms

Table 3.5: Biochemical test results of the isolates

Source of the isolates		Matrivandar sweet curd	Arong sour curd (light yellowish colony)	Arong sour curd (white colony)	Shakti sweet curd (white colony)	Shakti sweet curd (light yellowish colony)	Buttermilk (white colony)	Buttermilk (off-white colony)	Delicia sweet curd	Cauliflower (white colony)
Gram stain		+	+	+	+	+	+	+	+	-
Nitrate reduction		+	+	+	+	+	+	+	+	+
TSI	**Slant**	A	K	✖	A	✖	A	K	A	✖
	Butt	✖	A	A	A	A	A	✖	A	✖
	Gas	No	No	No	No	No	No	No	No	No
H_2S production		▬	▬	▬	▬	▬	+	▬	▬	+
Dextrose		▬	▬	▬	▬	▬	+	▬	+	▬
Catalase		▬	▬	▬	▬	▬	+	+	+	▬
Oxidase activity		▬	▬	▬	▬	▬	▬	▬	▬	▬
Growth at 15^0 c		▬	▬	▬	▬	▬	▬	▬	▬	▬
Motility		▬	▬	▬	▬	▬	▬	▬	▬	▬
Indole activity		▬	▬	▬	▬	▬	+	▬	▬	▬
Growth at 45^0 c		+	▬	▬	▬	▬	▬	▬	+	▬
Gelatin hydrolysis test		▬	▬	▬	▬	▬	▬	▬	▬	▬
Casein hydrolysis test		▬	▬	▬	▬	▬	▬	▬	▬	▬
Suspected organism using ABIS software		*Lactobacillus ingluvvei* or *Lactobacillus hordei*	*Lactobacillus composti*	*Lactobacillus composti*	*Lactobacillus composti*	*Lactobacillus composti*	*Lactobacillus composti*	*Lactobacillus composti*	*Lactobacillus composti*	*Lactobacillus composti*

Key notes: Acid (A), Alkaline (K), No change (✖), Positive (+), Negative (-)

Table 3.5 (Continued): Biochemical test results of the isolates

Source of the isolates		Cauliflower (light yellowish colony)	Turnip (white colony)	Turnip (light yellowish colony)	Carrot (white colony)	Carrot (light yellowish colony)	Brinjal (off-white colony)	Brinjal (white colony)	Cabbage (off-white colony)	Cabbage (white colony)	Cabbage (light yellowish colony)	Dhaka cheese
Gram stain		+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction		-	+	-	-	-	+	-	-	-	-	+
TSI	**Slant**	×	×	×	K	×	A	K	×	×	×	A
	Butt	×	×	×	A	×	A	A	×	×	×	A
	Gas	No	No	No	No	No	No	No	No	No	No	No
H_2S production		-	-	-	-	-	-	-	-	-	-	-
Dextrose		-	-	-	-	-	-	-	-	-	-	-
Catalase		-	-	-	-	-	-	-	-	-	-	-
Oxidase activity		-	-	-	-	-	-	-	-	-	-	-
Growth at 15^0 c		-	-	-	-	-	-	-	-	-	-	-
Motility		-	-	-	-	-	-	-	-	-	-	-
Indole activity		-	-	-	-	-	-	-	-	-	-	-
Growth at 45^0 c		-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis test		-	-	-	-	-	-	-	-	-	-	-
Casein hydrolysis test		-	-	-	-	-	-	-	-	-	-	-
Suspected organism using ABIS software		*Lactobacillus plantarum*	*Lactobacillus plantarum*	*Lactobacillus plantarum*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*	*Lactobacillus composti or Lactobacillus saniviri*

Key notes: Acid (A), Alkaline (K), No change (×), Positive (+), Negative (-)

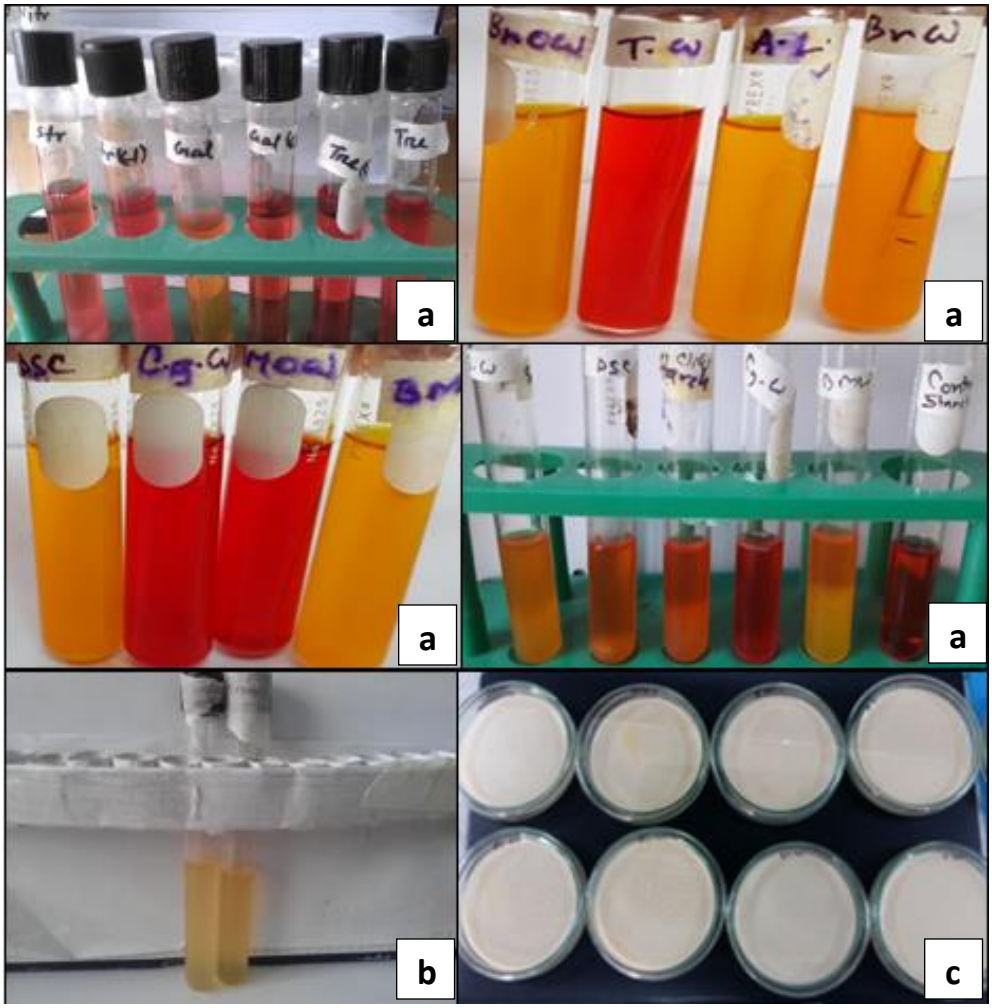


Figure 3.5: Results of biochemical characterization of LAB isolates

a. Fermentation of different carbohydrates, b. Result of nitrate reduction test, c. Casein hydrolysis test

Table 3.6: Bacterial identification analyzed by ABIS software based on biochemical tests (ABIS online)

No.	Source and colony colour of the isolates	Suspected organisms	Percentages of probability
1.	Matri Vandar Sweet Curd	*Lactobacillus ingluviei* or *Lactobacillus hordei*	87%
2	Arong Sour Curd (Light yellowish colony)	*Lactobacillus composti*	82%
3	Arong Sour Curd (white colony)	*Lactobacillus saniviri*or *Lactobacillus kalixensis*	85-87%
4	Shakti Sweet Curd (white colony)	*Lactobacillus composti*	82%
5	Shakti Sweet Curd (Light yellowish colony)	*Lactobacillus compost*ior *Lactobacillus salivarius*	82-86%
6	Buttermilk (white colony)	*Lactobacillus frumenti*	85%
7	Buttermilk (off-white colony)	*Lactobacillus ingluviei*or *Lactobacillus delbrueckii*	87-90%
8	Delicia sweet curd	*Lactobacillus oris*	82%
9	Cauliflower (white colony)	*Lactobacillus plantarum*	91%
10	Cauliflower (light yellowish colony)	*Lactobacillus salivarius*or *Lactobacillus equi*	86%
11	Turnip (white colony)	*Lactobacillus paucivorans*	86%
12	Turnip (light yellowish colony)	*Lactobacillus compost*ior *Lactobacillus saniviri*	83-86%
13	Carrot (white colony)	*Lactobacillus frumenti*	90%
14	Carrot (light yellowish colony)	*Lactobacillus mali*or *Lactobacillus antri*	84%
15	Brinjal (off-white colony)	*Lactobacillus ingluviei*	87%
16	Brinjal (white colony)	*Lactobacillus frumenti*	86%
17	Cabbage (Light yellowish colony)	*Lactobacillus ultunensis*	78%
18	Cabbage (offwhite colony)	*Lactobacillus paucivorans* *Lactobacillus ingluviei*	87-91%
19	Cabbage (white colony)	*Lactobacillus ingluviei*or *Lactobacillus paucivorans*	83-87%
20	Dhaka cheese	*Lactobacillus composti* *Lactobacillus mali*	81-83%

Chapter 4

Discussion

4. Discussion

4.1 Discussion

In this study, twenty bacteria isolates were suspected as LAB (Lactic Acid Bacteria) by biochemical tests (Cappuccino & Sherman, 2005) using ABIS online software isolated from various types of fermented foods such as yogurt, fermented vegetable residues and some other dairy products. LAB isolates were primarily identified as homo-fermentative lactobacilli, consist *Lactobacillus ingluviei*, *Lactobacillus composti*, *Lactobacillus frumenti*, *Lactobacillus salivarius* and *Lactobacillus paucivorans*. *Lactobacillus ingluviei* and *Lactobacillus composti were* the dominant members of the LAB population in the twenty isolates (Table: 3.5).

The twenty LAB isolates showed ZOI when the agar well diffusion method was carried out to find the antimicrobial activity of the isolates against seventeen (17) test organisms. According to the results of primary screening, the CFS (cell-free supernatants) of the LAB isolates showed antimicrobial effects against the indicator strains in this present work. Out of 20 LAB isolates, 12 were found to successfully inhibit *EPEC* (atypical) and *EPEC* (typical) respectively, 11 showed significant effect against *Enterobacter cloace,* 10 against *EAEC,* 9 worked against *Klebshiella sp., Salmonella typhi* & *Proteus vulgaris, 8 worked against Shigella flexneri, Streptococcus pyogens, Bacillus subtilis,* 7 worked against *ETEC* & *Shigella dysentry,* 6 were found to inhibit *Escherichia coli, 4 worked against Pseudomonas areuginosa & Streptococcus agalactiae, 3 workrd against Streptococcus pneumoniae,2 showed inhibition against Bacillus cereus.* From the results of well diffusion technique, it can be suggested that the presence of antimicrobial agents in the CFS of LAB isolates that inhibit the growth of the tested organisms. The probable cause of antimicrobial activity of the isolates might have resulted from extracellular proteins, the presence of lactic acid, hydrogen peroxide or other substances. In a relatively similar study, Siddique *et al.* (2012) isolated a total of 29 lactic acid bacteria from 5 different food samples. All of the isolates were then tested against 4 most frequently encountered pathogens, *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, and Vibrio cholerae*. Out of 29 isolates, 17 were found to successfully

inhibit *Escherichia coli*, 11 worked against *S. aureus*, 11 against *S. typhimurium*, and 13 showed significant effects against *V. cholerae.*

Lactic acid bacteria (LAB) belong to the phylum Firmicutes. LAB constitutes a highest percentage of bacteria that produce probiotic properties (Collins *et al.*, 1998), (Heinemann, 1908), (Schrezenmeir, 2001). Among compounds which are produced by Lactic acid bacteria during lactic acid fermentations are: organic acids, diacetyl, hydrogen peroxide and bacteriocins or other bactericidal proteins (Oyetayo*et al.*, 2003), (Rodriguez*et al.*, 2003), (Holzapfel*et al.*, 2001), (Yoshida *et al.*, 2003). Levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions (Lindgren & Dobrogosz, 1990).

The majority of bacteriocins from gram-positive bacteria come from lactic acid bacteria (Ennahar *et al.*, 2000), (Garneau *et al.*, 2002). Bacteriocin activity is more preferable for antimicrobial properties; however LAB that can provide any addition to inhibition of pathogens is also desirable (Vijarakumar & Muriana, 2015). Therefore, the isolates can be paired with other LAB isolates that do contain bacteriocin activity, because it can provide enhanced antimicrobial effects (O'Sullivan *et al.*, 2002). Since bacteriocins are proteins produced against closely related species (Klaenhammer, 1988).

On the other hand, hydrogen peroxide (H_2O_2) is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dehydrogenase (NADH). The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Ammor *et al.*, 2006), (Axelsson, 2004). 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).

And, it should 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). From these conducted studies, it can be seen that a good proportion of the entire sample sources may contain the target material: bacteriocin.

Since zone of inhibition (ZOI) was observed in agar diffusion method for all the isolates, it would mean that bacteriocin group might present even after using the supernatants only. Isoelectric focusing or multiple chromatographic separations, including cation exchange, gel filtration, hydrophobic interaction and reverse-phase liquid chromatography are necessary to achieve significant purification of bacteriocins. Usually the yields obtained are low. An ideal protocol for bacteriocin production should be one that is applicable to large-scale purification, leading to bacteriocin yields higher than 50% and purity around 90% (Schobitz *et al.*, 1999).

Selim *et al*. (2014) studied the viability of bacteria in seven probiotic products for animal production available in Bangladesh. The results of the study concluded that viability of bacteria in commercial probiotic products were not found at a minimum level and therefore may not be sufficient for colonization of the animal gut. Therefore, in this study, potential probiotic strains were subjected to isolate from the local food samples.

4.2 Conclusion

This study was targeted to screen out LAB isolates with antimicrobial property that contain extracellular proteins of bacteriocin group. Further study is needed to find the specific causes of antimicrobial property demonstrated by the LAB isolates. A bigger and more diverse sample collection should be chosen for the study and the number of isolates should be increased to alleviate the possibility of obtaining bacteriocin producing strains of LAB. Since bacteriocins are proteins produced against closely related species (Klaenhammer, 1988), to determine optimal parameters for the bacteriocin production, it is necessary to determine the ideal conditions of growth of the lactic strains and the composition of the culture medium.

Lactic Acid Bacteria can be used as probiotics against some food borne pathogens as the LAB isolates in this study demonstrated inhibitory properties. LAB can be also used as live cells as probiotics since they have a GRAS (koponen, 2004) status although inhibition by bacteriocin might be more effective as well.

Since antibiotic resistance is an alarming issue, it is expected that this experiment will contribute in the development of sustainable technology to produce natural products with antimicrobial properties.

Maximum effort will be given to get bacterial isolates which can produce high percentage of antimicrobial proteins and analyze which may provide data sufficient enough to produce industrially important enzymes.

Chapter 5

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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
Sodium acetate $3H_2O$	5.0
Tri-ammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Agar	10.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

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

T_1N_1

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

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 II

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.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of pdimethylaminobenzaldehyde (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.

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-phenylenediaminedihydrochloride 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 % H_2O_2

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 III

Instrument

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