Screening of Probiotics Activities of *Lactobacilli* Strains Isolated From Black Tiger Shrimp (*Penaeus monodon*)

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF FISHERIES, UNIVERSITY OF DHAKA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SICENCE IN FISHERIES

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DEDICATED

TO

MY BELOVED PARENTS

Acknowledgement

First of all, I want to express my gratefulness to the Almighty Allah who gave me physical, mental and spiritual energy to enable me to perform this research work and submit this paper.

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ABSTRACT

The objective of this study was to characterize probiotic *Lactobacillus* spp. isolated from black tiger shrimp (*Penaeus monodon*) focusing on their safety, antimicrobial and antibiotic susceptibility properties. Sixteen colonies of lactobacilli isolated from ten samples of shrimp were screened for their probiotic use. These isolates were identified and characterized on the basis of their morphological and biochemical test results. The IMViC, Catalase, Oxidase test status of selected isolates showed that these isolates were identified as *Lactobacillus* spp. Four isolates of lactobacilli were selected for acid and bile tolerance. These isolated strains were assayed for their susceptibility to twelve antibiotics such as ampicillin, penicillin G, gentamicin, tetracycline, erythromycin, vancomycin, neomycin, chloramphenicol, kanamycin, ciprofloxacin, doxycycline & nalidixic acid using the agar overlay disk diffusion method. Isolate-1 and Isolate-2 were resistance to five antibiotics among twelve antibiotics used. All the isolates were resistance to gentamicin, and ciprofloxacin but susceptible to ampicillin, tetracycline, erythromycin and chloramphenicol. For determination of antimicrobial activity, all the isolates were assayed against eight pathogenic test organisms using agar well diffusion method. Three isolates showed to inhibit all the test organisms though they vary in zone of inhibition diameter. The highest zone of inhibition was observed by isolate-1 against *E. faecalis* (19 mm), isolate-2 against *E. coli* (20mm) and isolate-4 against *Bacillus subtilis* (14 mm). All the isolates showed lowest activity against *B. megaterium* and *S. aureus*. A substantial zone of inhibition was found against *E. coli* & *E. faecalis*, which adds further support to the suggestion that the probiotic strain could help prevent intestinal infection. This study suggested that the indigenous microflora of shrimp itself is a potential source of probiotics.

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List of Abbreviations

hrs	Hour (s)
µl	Microliter
µM	Micromolar
mM	Milimolar
sp.	Species
U	Unit
UV	Ultra Violet
^{0}C	degrees Celsius
Cm	centimeter
et al.	and others
gm	grams
L	litre
M	molar
OD	optical density
µl	microlitre
mm	millimeter
mM	millimolar

1. INTRODUCTION

1.1 Background

Marine aquaculture is one of the most important agricultural industries of Bangladesh. Black tiger shrimp (*Penaeus monodon*) culture has increased sharply and commonly follows the semi intensive culture system because it produces higher yields than extensive culture systems. High mortality and low production of shrimp in Bangladesh resulting from environmental deterioration and the pathogen outbreaks were observed. Wastewater effluents from semi intensive shrimp farming generally contain a considerable amount of waste nutrients (ammonia, nitrate, nitrite and phosphate), organic matter and inorganic solid which contribute to eutrophication in receiving waters (Trott and Alongi 2000; Jackson *et al.* 2003). Moreover, effluents and organic sludge from shrimp ponds may contain pathogenic microorganisms such as *Vibrio sp.* and *Pseudomonas aeroginosa*.

Consequently, a wide range of antimicrobial substances (oxytetracycline, ciprofloxacin, nitrofurantoin, furazolidoneor chloramphenicol) have been applied to control and prevent disease outbreaks in hatcheries and farms. The negative effect of antibiotics in shrimp culture is the evolution of antibiotic-resistant microorganisms that can transfer resistance to pathogenic microorganisms, leading to a reduced efficiency of antibiotics in treating diseases (Kautsky *et al.* 2000). Sustainability of shrimp production requires suitable cultivation practices (Phillips *et al.* 1993; Primavera 1994, Ziemann *et al.* 1992).

Presently, probiotics are gaining popularity as environmentally-friendly alternatives for antibiotics in improving shrimp health and minimizing disease (Gatesoupe 1999; Senok *et al.* 2005). Probiotics are defined as microorganisms or microbial cell preparations with beneficial health effects on a host by controlling the balance of intestinal microorganisms, enhancing digestibility and absorption capacity as well as decreasing organic waste.

Although probiotic supplies a putative alternative to antimicrobial agents for shrimp cultivation, probiotics should be specifically selected to optimize their effectiveness. Commercial probiotics constitute single or multiple strains of beneficial bacteria. Among the potential probiotics, lactic acid bacteria (LAB) are reported to have important effects in animals. *In vivo* studies investigating health benefits of potential probiotics are time-

consuming and often expensive; hence, the consequent use of *in vitro* tests as selection criteria to reduce the number of strains and find the most effective organism is unavoidable. Colonization ability and antibacterial effects are major characteristics in the selection of LAB as probiotic candidates because these attributes provide competitiveness against enteric pathogens and reduce their colonization. The adherence capability of a bacterial strain to the digestive tract is presumably a prerequisite for colonization.

Recent studies have focused on improving the probiotic efficacy and represent more applicable supplements in animal nutrition. The beneficial effects of expression of α-amylase, phytase, β-glucanase, xylanase, and cellulase enzymes in the Lactobacillus show the importance of enzymatic activities in bacterial strains of probiotics (Forouhandeh *et al.* 2010; Hoque *et al.* 2010; Aymerich *et al.* 2006). A probiotic that has the enzymatic activities can improve digestion. Hence, a screening process that evaluates all of these bacterial attributes increases the likelihood of selection of an efficacious probiotic.

Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Helland *et al.* 2004). Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immune stimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance (Parvez *et al.* 2006). In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host, and prove safety for consumer (De-Vries *et al.* 2006). To survive in the gut, the organisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract. Besides, quality assurance programmes associated with research, development, production and validation of the health benefits of these bacteria require their relevant characterization and identification. Lactic acid bacteria can reduce the number of pathogens in the gastrointestinal tract by production of bacteriocins, H_2O_2, and especially organic acids (Chou, L.S and Weimer, B. 1999).

1.2 Rationale

Probiotic microorganisms that are generally used in aquaculture such as lactic acid bacteria (LAB) including *Lactobacillus acidophilus, L. bulgaricus, L. casei, L. plantarum,* and *L. rhamnosus* (Angulo, F. 2000). Some other bacterial species such as *Bacillus* sp. and *Bifidobacterium* sp. as probiotic strains also used in aquaculture (Balcázar, J.L. 2007).

Lactic acid bacteria (LAB) are very helpful in fermenting sugar into lactic acid for removal or deduction of germs in digestive system. This wonderful specie regarding bacteria is prosperous with health protecting features. In Medical Science, this splendid bacterium is doing superb performance intended for curing diarrhea throughout children and vaginal yeast infections (Ammor *et al.* 2007). This specie in human's digestive system is found obviously, but in circumstance of shortage, with some herbal foods or supplements it may be produced. For individual health, this style of conversing and protection bacteria is playing a tremendous role.

Plate 1: *Lactobacillus sp.*

Basically living items or objects experience quite a few health issues. For absolute curing and further prevention, doctors prefer *Lactobacillus* being an instrument or therapy. For digesting eaten food, perfect intake of nutrients in addition to elimination of sugar

cravings, this natural bacterium has become utilized by stomach as well as digestive mechanism.

Lactobacillus found in prosperous quantity in eating products like milk plus some natural vegetables. It exists using some dairy items, which give lots of these fertile germs. In case of shortage of this digestive element, one can choose these sources with perfect consultation of the medical experts. Excess amount regarding *Lactobacillus* can boost gas in belly that creates several casual in addition to serious problems such as chronic constipation, piles and bowel attacks. But luckily, having accurate diagnosis in addition to treatment, all these infections is usually reduced or manipulated.

1.3. Research Need

In recent decades, prevention and control of animal diseases has focused on the use of chemical additives and veterinary medicines, especially antibiotics, which generate significant risks to public health by promoting the selection, propagation and persistence of bacterial-resistant strains (Balcazar *et al*. 2003).

There is a lack of information about probiotic bacteria in Bangladesh. It is also essential to understand the mechanisms of action in order to define selection criteria for potential Probiotics. Therefore, more information on the host/ microbe interactions *in vivo*, and development of monitoring tools (e.g. molecular biology) are still needed for better understanding of the composition and functions of the indigenous microbiota, as well as of microbial cultures of ‘‘probiotics’’. The use of probiotics is an important management tool, but its efficiency depends on understanding the nature of competition between species or strains. So our study is essential for the probiotic characterization of *Lactobacillus* sp. isolated from black tiger shrimp focusing on their safety, antimicrobial and cholesterol-lowering properties and further select the most suitable strain of LAB from the shrimp for potential application as a probiotic supplement with regard to feasibility and sp.eed of methods.

1.4. Aims and Objectives of the Study

The aim of the present study was to isolate probiotic bacteria from shrimp samples and to determine the antibacterial potential of the isolated probiotics. The plan for present research investigation was design to fulfill the following objectives:

- Isolation of probiotic bacteria from shrimp.
- Biochemical confirmation of the isolates and comparison with reference strains.
- To determine the potential of isolated probiotic bacteria to inhibit the growth of common pathogenic bacteria.

2. MATERIALS AND METHODS

2.1. Research Location

The experiments were conducted at Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Kudrat-E-Khuda Road, Dhanmondi, Dhaka-1205.

2.2. Sampling Techniques

2.2.1. Sampling site and Collection of sample

For present study, ten (10) samples were collected from local markets of Dhaka. During sample collection sterile paper bag, marker pen, note book etc were taken to the sites for the purpose of sampling. The whole sampling procedure was conducted under aseptic condition.

Table- 2.1: Serial no, sampling date, sample ID and sampling place

Serial No.	Sampling Date	Sampling ID	Source of sample
1	30.11.13	S-1	Satkhira
2	03.12.13	S-2	Satkhira
3	10.12.13	S-3	Satkhira
4	10.12.13	S-4	Satkhira
5	12.12.13	S-5	Bagerhat
6	12.12.13	S-6	Bagerhat
7	12.12.13	S-7	Bagerhat
8	15.12.13	S-8	Bagerhat
9	15.12.13	S-9	Bagerhat
10	15.12.13	S-10	Bagerhat

2.2.2. Transportation of Sample

After collection, shrimp sample were transported in sterile paper bag which remain in sterile box and maintained in aseptic condition.

2.2.3. Preservation of Sample

Samples were brought to the laboratory and carefully preserved in the refrigerator at 4°C for culture and microbiological analysis.

2.3. Media Used for Probiotic Bacteria Isolation

Lactobacillus spp. were isolated from shrimp samples by using selective MRS broth and MRS agar media (De Man, Rogosa and Sharpe 1960). Additionally 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of lactobacillus (Hartemink *et al.* 1997). The pH of the media was adjusted to 6.4±.2.

De Man, Rogosa and Sharpe (MRS) Medium	gm/l
Peptone	10g
Meat extract	10 g
Yeast extract	5 g
Glucose	20 g
K_2HPO_4	2 g
Ammonium citrate	2 g
$MnSO_4.4H_2O$	0.05 g
Magnesium sulfate	0.10 g
Sodium acetate	5 g
Tween-80	1 ml

The pH was adjusted to 6.4± 0.2 before autoclaving at 121°C for 15 min.

2.4. Isolation Techniques of Probiotic Bacteria

Probiotic organisms were isolated from shrimp samples. Eight different samples were tested. 10 g of each shrimp sample was separately dissolved in 90 ml of sterile phosphate buffer saline.

- The Stromacher was used to mix the sample.
- The samples were then enriched in MRS broth and plated into MRS agar medium by swabbing to determine the growth of the probiotics.

- The plates were incubated anaerobically using Gas Pak jars at 37°C for 72 hours.
- The isolated organisms were examined according to their colony morphology, catalase, oxidase and gram staining.
- Stock cultures of the probiotics are maintained in their respective culture media at 4°C.

2.5. Identification Techniques

2.5.1. Observation

All the isolates from shrimp samples were examined under Bright Field microscope.

2.5.2. Macroscopic View

The probiotic isolates from shrimp samples were appeared as white to cream colored, convex, circular colony after 3 days of incubation. For microscopic observation of all the isolates, Gram staining was done.

2.5.3. Morphology Based Techniques

2.5.3.1. Gram Staining

The gram reaction of the isolates was determined by light microscopy after gram staining. Cultures were grown in appropriate medium at 37°C for 24 h anaerobic conditions. Cells from fresh cultures were used for gram staining. The individual colony picks up aseptically into the agar plate with loops and smeared onto the slide and air dry or heat fixed. Crystal violet was added for 30 sec and rinsed with water (5 sec). Then grams iodine solution was added for 1 min and again rinsed with water (5 sec). Further, decolorizer (95% ethanol) was added for 15-30 sec and rinsed with water for 5 sec. Finally safranine was added for 60-80 sec and rinsed with water. Then, under light microscopy gram positives and purified isolates were determined. Gram positive cells will stain purple while gram negative cells stain pink/red (Ennis 2008).

2.5.4. Biochemical Studies of the Selected Isolates

2.5.4.1. Catalase Test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and cause gas bubbles. The formation of gas bubbles determines the presence of catalase enzyme and indicates the positive result (Holt *et al.* 1994).

$$2H_20_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Catalase test was performed to isolate in order to see their catalase reactions. For this purpose, two methods can be applied. Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h, 3% hydrogen peroxide solution was dropped onto randomly chosen colony. Also fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were chosen since LAB are known as catalase negative.

2.5.4.2. Oxidase Test

The enzyme oxidase, present in certain bacteria catalyses the transport of electron from donor bacteria to the redox dye tetra-methyl-p-phenylenediamine dihydrochloride. The dye in the reduced state has a deep purple color. Inoculated MRS plates were incubated anaerobically at 37°C for 76 hours. One colony of each isolates was semeared to filter paper already impregnated with oxidase reagent (1% tetra-methyi-p-phenylenediamine dihydrochloride). Basically this is a test to see if an organism produces cytochrome C oxidase. The positive result was indicated by the production of dark blue color within 7 seconds (MacFaddin, J.F.,Williams and Wilkins 1980).

2.5.4.3. Indole Test

10 ml tryptophan medium were poured in each McCartney bottle, after the media were autoclaved (for 15 minutes in 15 Ibs pressure at 121°C) inoculation processes were done. Then the inoculated media were left for incubation 24 hours at 37°C. *E coli* ATCC 8739 used as a positive control and a lab isolates of *Pseudomonas aeruginosa* was used as a negative control. The amino acid tryptophan can be broken down by enzyme tryptophanase to form Indole, pyruvic acid and ammonia as end products. Tryptophanase

differentiates indole-positive enterics (e.g, *E. coli*) from closely related indole-negative enterics. Indole can be detected with Kovac's reagent (indole reagent). (MacFaddin, J.F.,Williams and Wilkins, 1980).

2.5.4.4. Methyl-red Test

10 ml MR-VP broth was taken in each McCartney bottle and autoclaved (for 15 minutes in 15 Ibs pressure at 121°C) for methyl red (MR) and Vogas-Proskauer (VP) tests. After this, isolates were inoculated into MR-VP broth with a sterile transfer loop. The McCartney bottles were then incubated at 37°C. After 48 hours of incubation, the MR-VP broth of each bottle was equally split into two McCartney bottles (one of this bottles used for the MR test; the other was used for VP test). Then the bottles containing MR-VP broth that was subjected to MR test were incubated for another 24 hours. After this, five drops of the pH indicator methyl red was added to each bottle. The bottles were gently rolled between the palms of the hand to disperse the methyl red. In this test, *E. coli* ATCC 8739 used as a positive control and lab isolates *Pseudomonas aeruginosa* was used as negative control.

The methyl red test was used to identify enteric bacteria based on their pattern of glucose metabolism. Enterics that subsequently metabolize pyruvic acid to other acids lower the pH of the medium to 4.2. At this pH, methyl red turns red. A red color represents a positive test. Enterics that subsequently metabolize pyruvic acid to neutral end products lower the pH of the medium to only 6.0. At this pH, methyl red is yellow. A yellow color represents a negative test. (MacFaddin, J.F., Williams and Wilkins 1980)

2.5.4.5. Vogas - Proskauer (V.P.) Test

As previously mentioned in the MR test, 10 ml MR-VP broth was taken in each McCartney. The McCartney bottles were then incubated at 37°C for 48 hours. After incubation, 40% KOH (Baritt's reagent B) were added. After this, the bottles were shaken vigorously and allowed to stand for 20 minutes. *E. coli* ATCC 8739 used as negative controls and *Bacillus cereus* was used as positive control. (Voges and Proskauer 1898; Lennette *et al.* 1985).

2.5.4.6. Citrate Utilization Test

A loopful of each isolates was streaked onto citrate agar slant and then incubated for a maximum of 96 hours. The citrate test determines the ability of microorganisms to use citrate as the sole of carbon and energy. In this test, *E. coli* ATCC 8739 used as a negative controls and lab isolates *Pseudomonas aeruginosa* was used as positive control.

Simmons citrate agar, a chemically defined medium with sodium citrate as the carbon source, NH_4^+ as nitrogen source and the pH indicator bromothymol blue, is commonly used for this test. When microorganisms utilize citrate, they remove the acid from the medium, which raises the pH and turns the pH indicator from green to blue indicates that the microorganisms tested can utilize citrate as its only carbon source. (MacFaddin, J.F., Williams and Wilkins 1980)

2.5.4.7. Sugar Fermentation Test

Materials: Phenol red 0.0018 %

10% Sugar solution 1 ml of each (Filtered sterilized)

An overnight culture in MRS broth

Durham tube

MRS broth

P^H 6.5 and sterilization was done at 121°C for 15 min

MRS broth at p^H 6.5 was taken into each McCartney tube and phenol red (0.018 g per L) was added into the tube as p^H indicator. After autoclaving the medium (at 121°C for 15 min) 1 ml different types of sugar solutions (10%) (Filtered sterilized) were inoculated into the different tube. Then 200 µL an overnight liquid culture medium inoculated into the broth medium incubated anaerobically at 37°C for 24 h. If fermenting bacteria are grown in a liquid culture medium containing the carbohydrates they may produce organic acids as by-products of the fermentation. These acids are released into the medium and lower its p^H. If a p^H indicator such as phenol red or bromocresol purple is included in the medium, the acid production will change the medium from its original color to yellow.

Gases produced during the fermentation process can be detected by using a small, inverted tube, called a Durham tube (named after Herbert Edward Durham, English

bacteriologist, 1866-1945), within the liquid culture medium. After adding the proper amount of broth, Durham tubes are inserted into each culture tube. During autoclaving, the air is expelled from the Durham tubes, and they become filled with the medium. If gas is produced, the liquid medium inside the Durham tube will be displaced, entrapping the gas in the form of a bubble.

2.6.1. Acid Tolerance

For the determination of acid tolerance of the isolates, 100 µl overnight culture of the isolates were inoculated into MRS broth with varying pH ranging from 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The inoculated broths were then incubated in anaerobic condition for 24 h at 37°C. Growth of the bacterial isolates was measured using a spectrophotometer at 560 nm.

2.6.2. NaCl Tolerance Test

For the determination of NaCl tolerances of isolated lactobacillus, 10 test tube containing MRS broth were adjusted with different gradually increasing concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh overnight culture of lactobacillus. The initial optical density of the broth was measured at 600 nm by spectrophotometer. The broth was then incubated anaerobically overnight at 37°C. After 24 h of incubation, optical density of the culture broth was measured again at 600 nm. Growth of the probiotic bacteria was determined by comparing optical density with that of control broth (0%) (Graciela *et al.* 2001).

2.6.3. Bile Salt Tolerance

The *lactobacillus* isolates cultures were grown for 6h in MRS broth at 37°C. An aliquot of 1ml of the 6h old culture was inoculated into 100ml MRS broth with 0.2 or 0.4% (w/v) bile salts. Bacterial growth was monitored by determination of optical density at 650nm after 6 and 24h incubation period at 37°C. The percent difference between the variation of optical density (DO) of culture without bile salts ($\Delta D_{0\% BS}$) and the variation of optical density of culture containing 0.2 or 0.4% bile salts ($\Delta DO_{0.2 \text{ or } 0.4\% BS}$) would give an index of isolates surviving that can be expressed as follows:

$$\text{Surviving (\%)} = \frac{\Delta DO\ 0\%\ BS\ - \Delta\ DO\ 0.2\%\ or\ 0.4\%\ BS}{\Delta DO 0\% BS}$$

Classification criteria included four arbitrary level of bile salt tolerance: excellent if the isolate survived at 0.4% bile salt after 24h; very good if the isolate survived at 0.4% bile salt after 6h but not after 24h; good if the isolate survived at 0.2% bile salt after 24h but not at 0.4% bile salt; poor if the isolate did not survive in any experimental condition. An isolate survived if it demonstrated a surviving percentage equal or greater than 50% (Dora and Glenn 2002).

2.7. Assay for Determination of Gastric Juice Tolerance

2.7.1. Preparation of artificial gastric juice (for 1L)

NaCl (2 g), pepsin (3.2 g) were adjusted at a final pH with HCl without dilution and taken to 1L with distilled water. As a control, artificial gastric juice was adjusted at a final pH 6.6 with 5N NaOH. Sterilization was done by filtration (filter membrane 0.22 µm).

The Lactobacilli under study were grown in MRS broth at 37°C for 16 h. Then the artificial gastric juice having pH 2.22 and pH 6.6 were inoculated with the 2% (v/v) bacterial suspension. Both the media were incubated at 37°C and samples were taken at 0, 1, 2, 3, and 4h and after 24h for cell viability measurement. Optical densities were measured at 600 nm.

2.8. Antibiotic Susceptibility Test:

Susceptibility testing was based on the agar overlay disc diffusion test described by Charteris *et al.* as modified by Aymerich *et al*. Briefly, *Lactobacillus* strains were grown overnight in MRS broth at 37ºC under anaerobic conditions. Eight ml of MRS soft agar kept at 50ºC were inoculated with 200 µL of the grown culture. Petri dishes containing 15 mL of MRS were overlaid with 8.2 mL of the inoculated MRS and allowed to solidify at room temperature. Antibiotic discs were placed onto the overlaid plates and all plates were incubated at 37ºC for 24 h under anaerobic conditions. All isolates were screened for their susceptibility to penicillin G (10 µg), ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), erythromycin (15 µg), kanamycin (30µg) gentamicin (10 µg),

neomycin (10 µg), ciprofloxacin (5 µg), doxycycline (30 µg), nalidixic acid (30 µg) and chloramphenicol (30 µg). Inhibition zones diameters of antibiotics were compared to those defined by Charteris *et al*. for lactobacilli.

2.9. Antimicrobial Activity Test

Agar well diffusion method was modified (Schillinger and Lucke, 1989; Osuntoki *et al.* 2008) and used to detect antimicrobial activities of CFSs produced from Lactobacilli strains. These assays were performed in duplicate. The plates were poured with 20 mL nutrient agar media. The pathogenic strains were grown in nutrient broth for 24 hours and spread on the surface of nutrient agar plate. Four wells in each plate of 4 mm in diameter were cut into these agar plates by using a sterile tip and 30 µL of the CFSs of the isolates were placed into different well. The plates were pre-inoculated at 4°C for the diffusion and incubated aerobically overnight at 37°C. The plates were examined for zones of inhibition.

3. RESULTS

3.1. Isolation & Characterization of *Lactobacillus* Bacteria

3.1.1. Morphological appearance of colonies

A total of sixteen (16) isolates were selected after pre-enrichment & enrichment. These isolates were produced white pigmentation on MRS agar (Plate- 3.1).

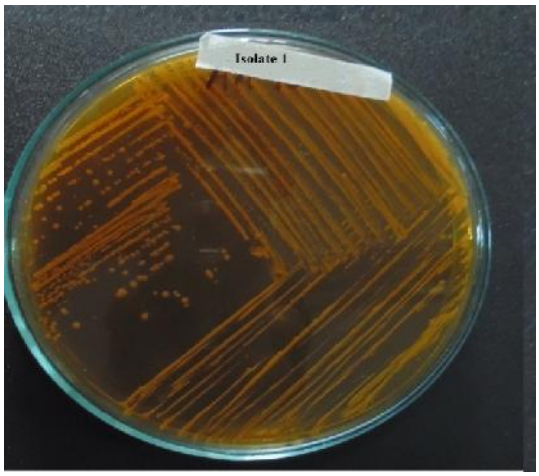

Plate - 3.1(a): Isolate – 1

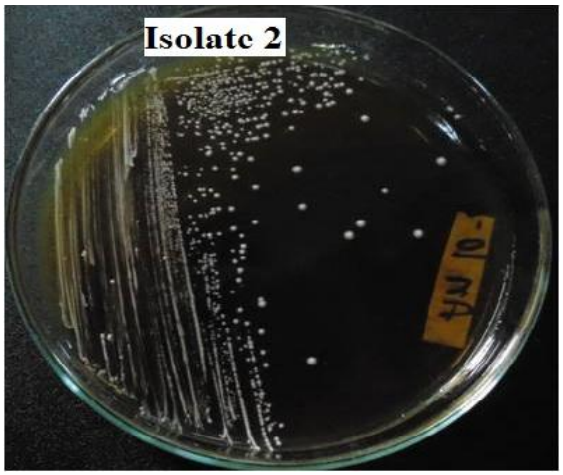

Plate - 3.1(b): Isolate - 2

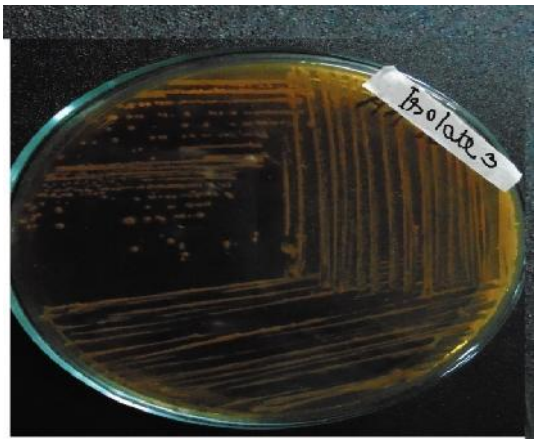

Plate - 3.1(c): Isolate – 3

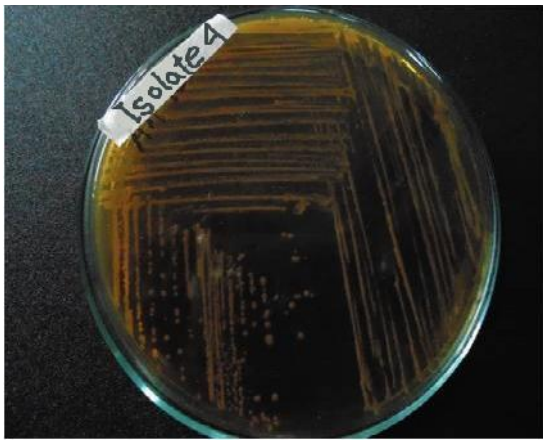

Plate - 3.1(d): Isolate – 4

Plate - 3.1 Growth of *Lactobacillus* on MRS media plate

Table- 3.1: Morphological Characterization of the Isolates

Morphological Characteristics	Isolate – 1	Isolate - 2	Isolate – 3	Isolate – 4
Colony color	Dull white	Shiny white	Creamy white	Creamy white
Colony margin	Entire	Entire	Irregular	Irregular

3.1.2. Microscopic Observation

All the isolates were examined under bright field microscope to observe their morphological features. They showed similar staining pattern. These were gram positive, rod shaped bacteria. Gram positive cell retain the crystal violet-iodine complex.

Microscopic views of the isolates are shown in the following figures:

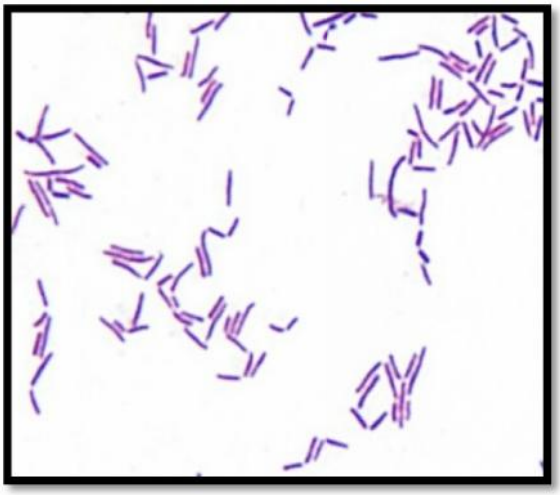

Plate- 3.2(a): Isolate - 1

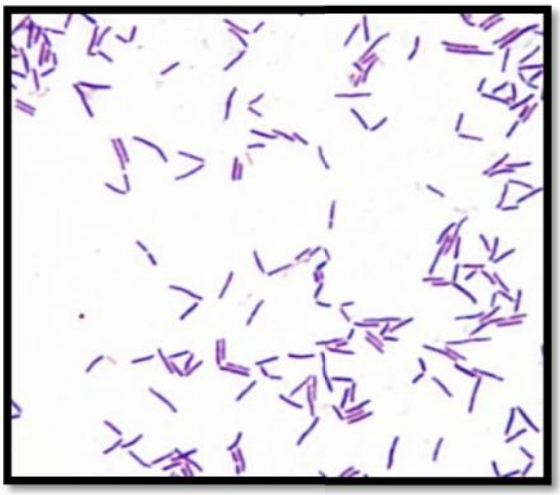

Plate- 3.2(b): Isolate - 2

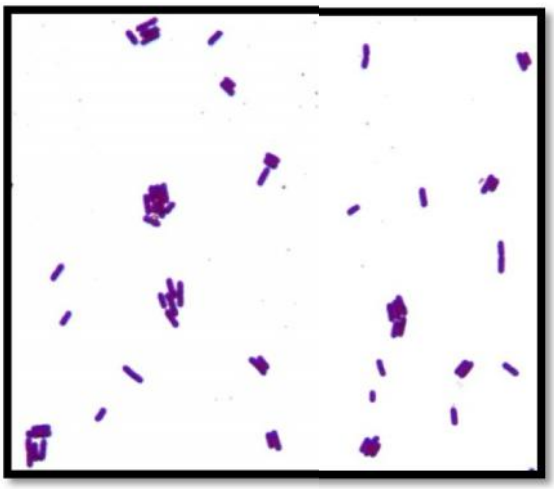

Plate- 3.2(c): Isolate – 3

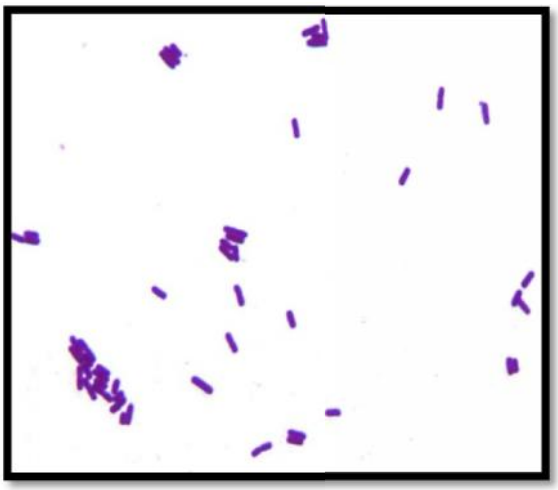

Plate- 3.2(d): Isolate - 4

Plate- 3.2: Microscopic observation of isolates after gram staining

Morphological Characteristics	**Isolate – 1**	**Isolate - 2**	**Isolate – 3**	**Isolate – 4**
Cell morphology	Long rods	Long rods	Medium to short rods	Medium to short rods

3.1.3. Biochemical Characterization of the Isolates

3.1.3.1. Indole Test

After addition of kovac's reagent, a positive result had a red layer at the top due to the production of indole and negative result has a yellow/brown layer. All the presumptive isolates were indole negative. The lab isolates *E. coli* ATCC 8739 used as a positive control.

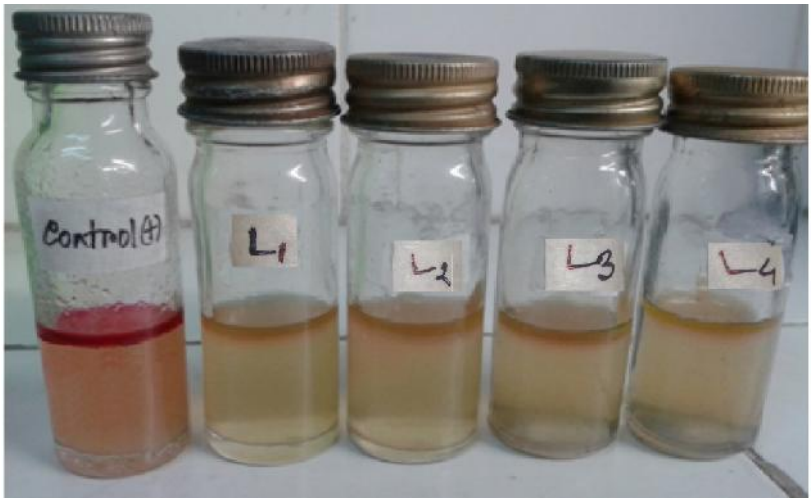

Plate - 3.3: Indole test

3.1.3.2. Methyl Red Test

Five drops of the pH indicator methyl red were added to the tubes. The tubes were gently rolled between the palms of the hands to disperse the methyl red. In all isolates, a brown color represented the negative test. The lab isolates *Escherichia coli* ATCC 8739 was used as positive control and red color appeared.

Plate- 3.4: Methyl red test

3.1.3.3. Voges - Proskauer Test

The addition of 40% KOH and 5% alpha-napthol revealed the absence of acetoin. All the isolates were VP negative.

Plate - 3.5: VP test

3.1.3.4. Citrate Utilization Test

A positive result for this test was indicated by color change in the medium from green to blue. When microorganisms utilized citrate, they removed the acid from the medium, which raised the pH and turned the pH indicator (bromothymol blue) from green to blue. Negative results were indicated by no growth and no color change. All the sample isolates were citrate negative.

Plate -3.6: Citrate utilization test (blue indicates positive result and green indicates negative results)

3.1.3.5. Catalase Test

All isolates were catalase negative. No bubbling occurs. The lab isolates of *Escherichia coli* was used as positive control.

3.1.3.6. Oxidase Test

An oxidize test was used because it allowed determining if the microorganisms contained the enzyme cytochrome oxidase, which transfers electrons to oxygen in the electron transfer chain (Wikipedia, 2008). An artificial electron donor (tetramethyl - p - phenylenediamine) was used as reagent. Cytochrome oxidase–negative bacteria did not cause any change in color. All isolates were oxidase negative.

Table - 3.2: Results of IMViC test, Catalase and Oxidase tests

Test isolates	Indole	Methyl red	VP	Citrate	Catalase	Oxidase
Isolate – 1	-	-	-	-	-	-
Isolate – 2	-	-	-	-	-	-
Isolate – 3	-	-	-	-	-	-
Isolate – 4	-	-	-	-	-	-

3.1.3.7. Sugar Fermentation Test

In the sugar fermentation patterns, mainly acid and gas production was observed. The sugar fermentation results are presented in Table- 4.3.
The sugar fermentation test results of all four isolates are shown in the following figures:

Plate - 3.7: Sugar fermentation pattern of isolate 1 and 2.

Plate-3.8: Sugar fermentation pattern of isolate 3 and 4

Table-3.3: Sugar fermentation patterns of the four isolates

Carbohydrate	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Glucose	+	+	+	+
Sucrose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Fructose	+	+	+	+
Arabinose	-	+	+	+
Galactose	+	+	+	+
Dextrose	+	+	+	+
Ribose	+	+	-	+
Mannitol	+	-	+	+

Rhamnose	+	-	-	-
Salicin	+	+	-	+
Cellobios	+	+	+	+
Xylose	-	+	+	+
Starch	-	-	-	-

N.B.: (+) means good fermentation, (-) means no fermentation

Sugar fermentation test results showed that all the four isolates were able to ferment glucose, sucrose, lactose, maltose, ribose, galactose, dextrose and cellobios, and were unable to ferment starch. Isolate- 4 showed highest fermenting ability as it was fermented all the sixteen sugars used except rhamnose and starch. Isolate- 3 showed almost similar sugar fermentation pattern like isolate- 4 but it was unable to ferment Salicin, rhamnose and starch. On the other hand, isolate- 1 and 2 showed different sugar fermentation pattern than isolate- 3 and 4. Isolate-1 was unable to ferment arabinose, sorbitol, xylose and starch, and isolate- 2 were unable to ferment mannitol, rhamnose, sorbitol and starch.

3.2. NaCl Tolerance of the Isolates

To determine the NaCl tolerance of the isolates, they were grown in medium containing gradually increasing concentrations of NaCl. After overnight incubation, optical density was measured at 600nm and the data was plotted to generate the following figure. It can be interpreted that all the isolates can grow up to 7% NaCl and can tolerate up to 9% NaCl. This type of high salt tolerance is a desirable property for a isolate to be used as probiotic.

Table- 3.4: Tolerance and growth* of isolates at different NaCl concentration

NaCl concentration	Isolate 1	Isolate 2	Isolate 3	Isolate 4
1%	++	++	++	++
2%	++	++	++	++
3%	++	++	++	++
4%	++	++	++	++

5%	++	++	++	++
6%	+	++	+	++
7%	+	+	+	++
8%	+	+	+	+
9%	+	+	-	+
10%	-	-	-	-

*Maximum growths were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth as negative sign (-) for NaCl.

3.3. Bile Salt Tolerance of the Isolates

Surviving percentage of lactobacilli isolates in MRS broth supplemented with 0.2% or 0.4% bile salts after 6h and 24h at 37°C.

Table -3.5: Bile salt tolerance of four isolates

Isolates	0.2% BS		0.4% BS	
	6h	24h	6h	24h
Isolate 1	82.45	79.56	73.55	62.89
Isolate 2	54.23	59.75	50.33	45.12
Isolate 3	51.73	57.88	49.63	43.67
Isolate 4	80.94	77.43	68.54	57.37

BS = Bile Salt

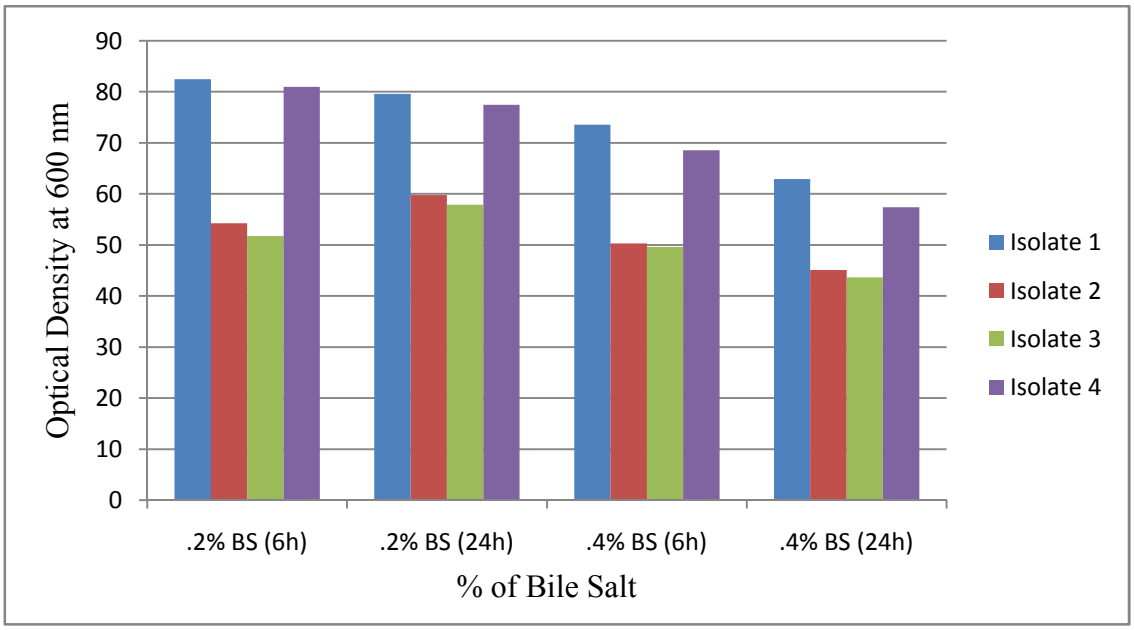

Figure -3.1: Bile salt tolerance of four isolates

All isolates demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C. These isolates were further investigated for their safety properties including sensitivity to antibiotic, antagonistic activity to common pathogen.

3.4. Assay for Determination of Gastric Juice Tolerance

Gastric juice tolerance of all the isolates was done at pH 2.2 and also at pH 6.6 as control. The results indicate that there were no losses of viability of cell in simulated gastrointestinal condition in case of artificial gastric juice pH 2.2. On the other hand, the present experimental results showed an optimum variation which indicates that cells were able to survive and may have little multiplication abilities in artificial gastric juice at pH 6.6. All the isolates containing media were incubated at 37°C and samples were taken at 0, 1, 2, 3, and 4h and after 24h for cell viability measurement. Optical densities were measured at 600 nm by spectrophotometer and the data were shown in following tables (Table- 4.5 and 4.6)

Table- 3.6: Gastric juice tolerance of the isolates at pH 2.2

Name of the isolates	Incubation Time (h)					
	0	2	4	6	8	24

1	0.417	0.397	0.322	0.182	0.113	0.076
2	0.337	0.283	0.2221	0.165	0.145	0.073
3	0.321	0.354	0.378	0.378	0.234	0.113
4	0.356	0.311	0.257	0.198	0.129	0.103

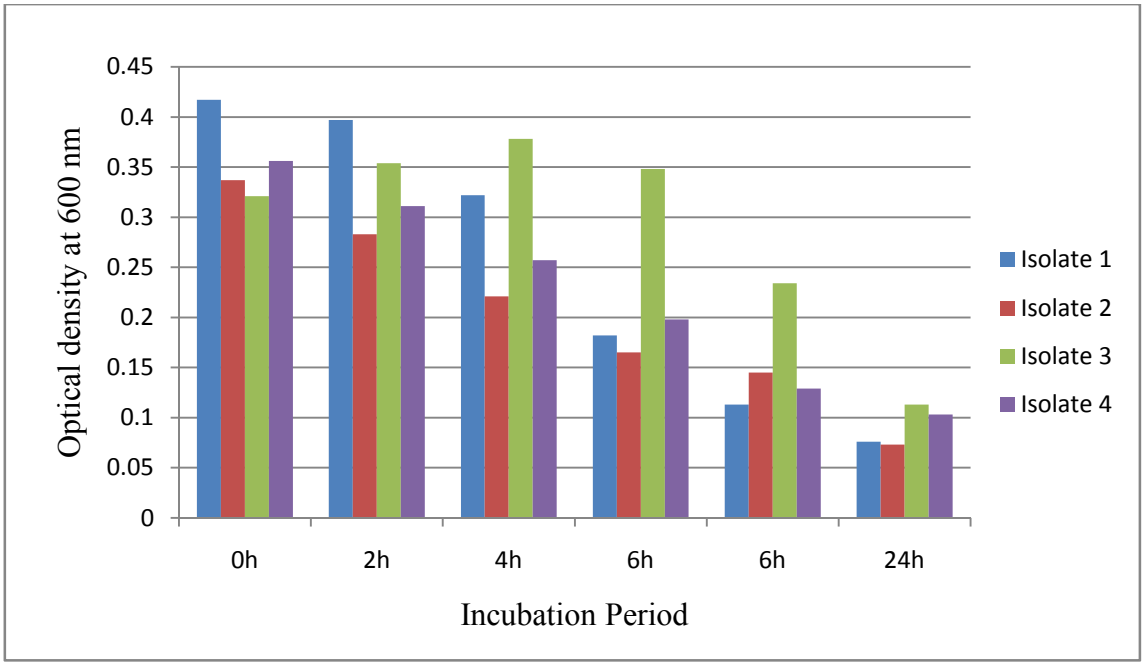

Figure- 3.2: Gastric juice tolerance of the isolates at pH 2.2

Table- 3.7: Gastric juice tolerance of the isolates at pH 6.6

Name of the isolates	Incubation Time (h)					
	0	**2**	**4**	**6**	**8**	**24**
1	0.576	0.587	0.543	0.298	0.214	0.712
2	0.385	0.217	0.298	0.245	0.274	0.323
3	0.402	0.365	0.323	0.387	0.432	0.605
4	0.465	0.412	0.332	0.376	0.245	0.443

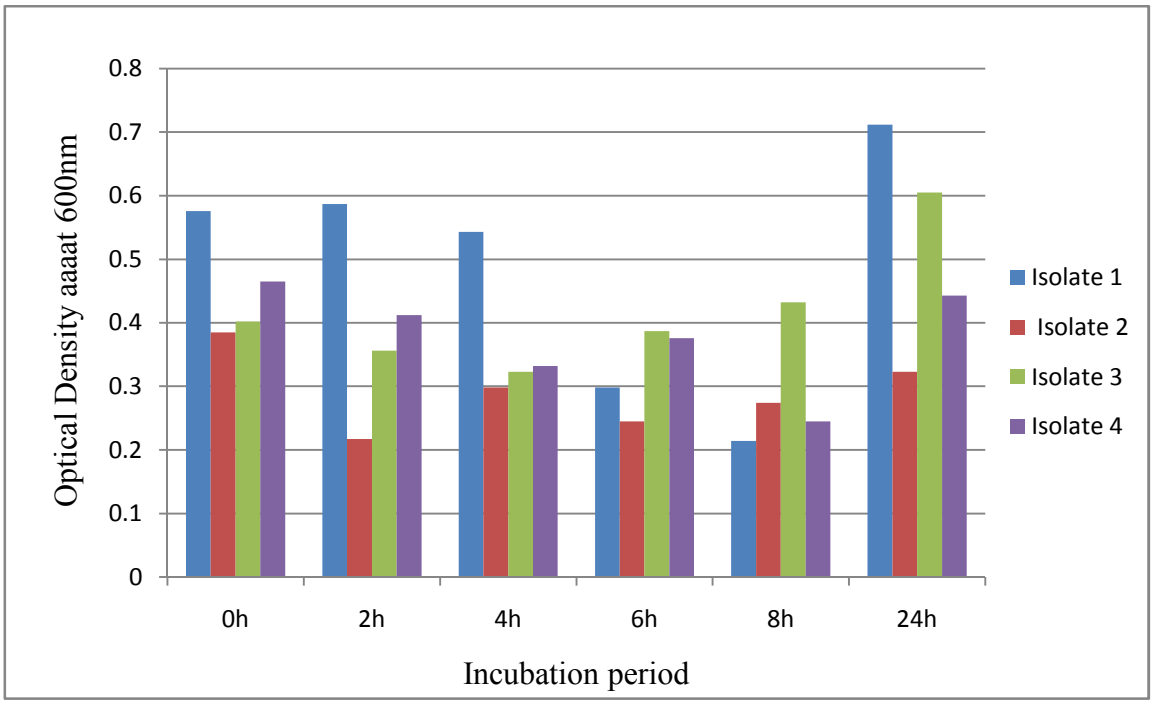

Figure- 3.3: Gastric juice tolerance of the isolates at pH 6.6

3.5. Antibiotic Susceptibility Pattern of the Isolates

All *Lactobacillus* strains isolated from shrimp samples were assayed for their susceptibility to twelve antibiotics, using the agar overlay disk diffusion method. Zone diameters were measured and strains were classified as susceptible (S), moderately susceptible (MS) and resistant (R). All these data are represented in Table-4.7 and results showed in following figures.

Table- 3.8: Antibiotic susceptibility pattern of all the isolates.

Antibiotic disk	Isolate-1	Isolate-2	Isolate-3	Isolate-4
Ampicillin (10)	S	S	S	S
Penicillin G (10)	R	MS	MS	S
Gentamicin (10)	R	R	R	R

Tetracycline (30)	S	S	S	S
Erythromycin (15)	S	S	S	S
Vancomycin (30)	MS	R	R	R
Neomycin (10)	R	S	MS	MS
Chloramphenicol (30)	S	S	S	S
Kanamycin (30)	R	MS	MS	R
Ciprofloxacin (5)	R	R	R	R
Doxycycline (30)	MS	R	S	S
Nalidixic acid (30)	MS	R	R	R

N.B.: Susceptible = S, Moderately susceptible = MS and Resistance = R.

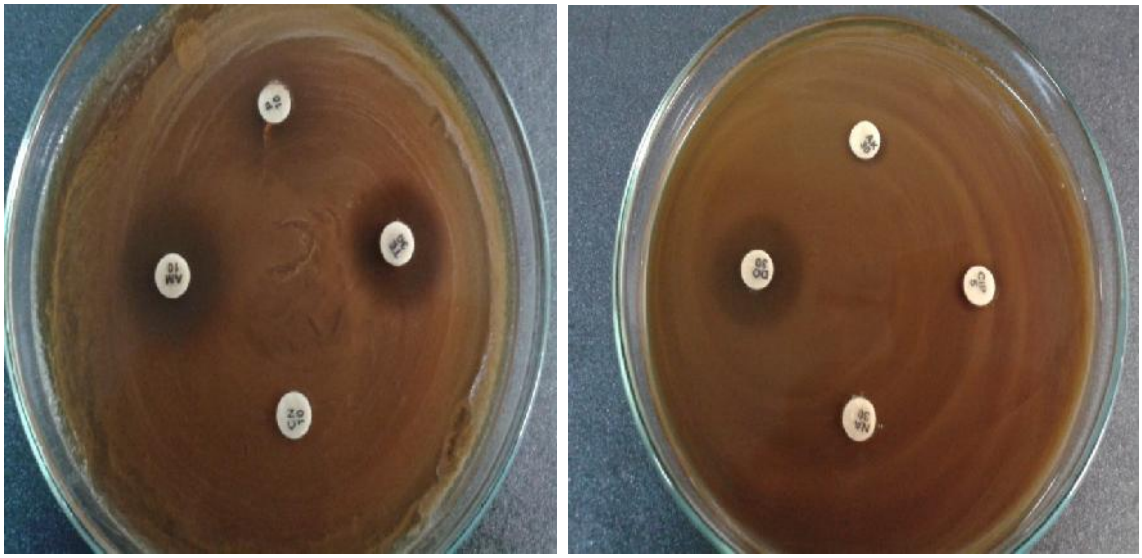

Plate-3.9: Antibiotic susceptibility pattern (zone of inhibition) of Isolate 1

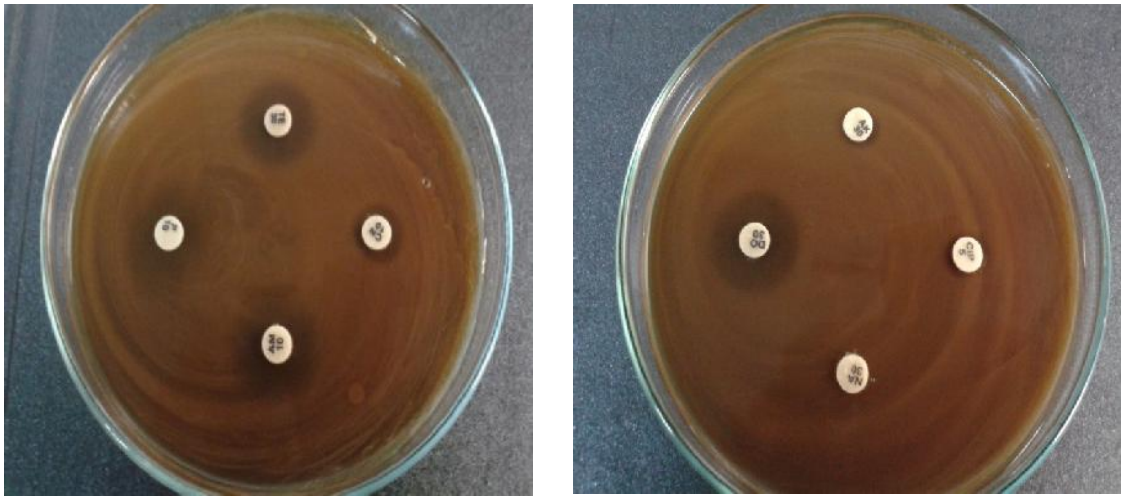

Plate-3.10: Antibiotic susceptibility pattern (zone of inhibition) of Isolate 2

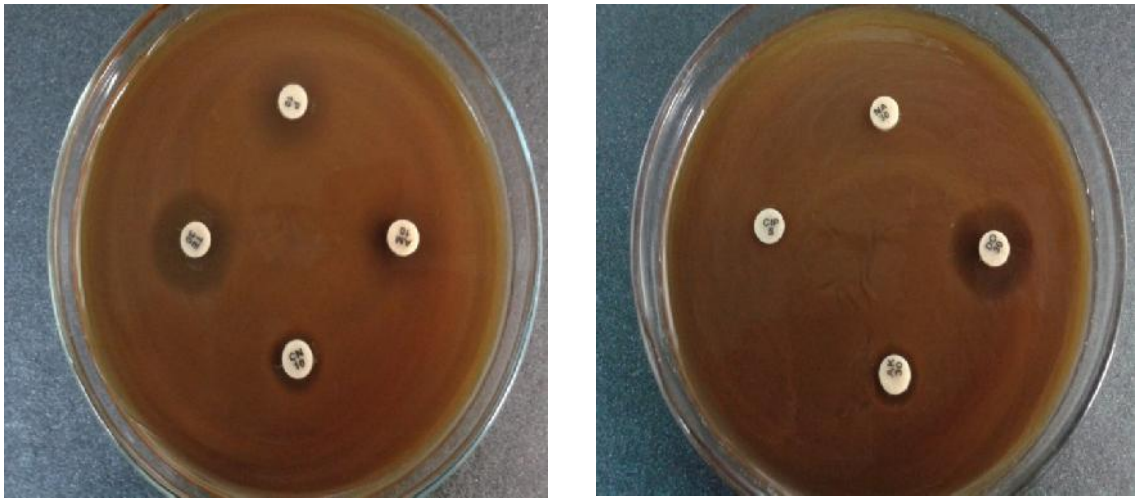

Plate-3.11: Antibiotic susceptibility pattern (zone of inhibition) of Isolate 3

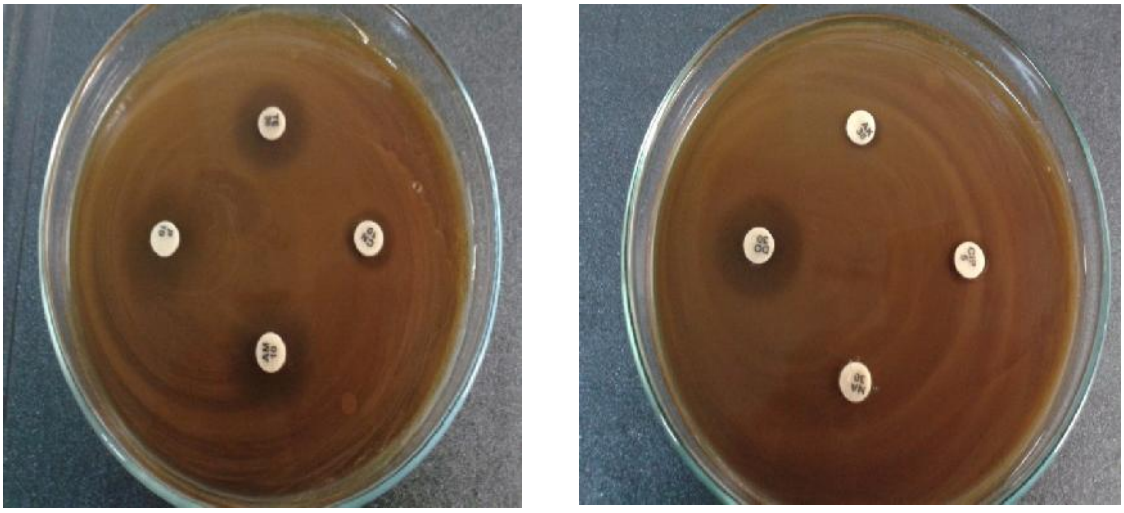

Plate-3.12: Antibiotic susceptibility pattern (zone of inhibition) of Isolate 4

4.6. Determination of Antimicrobial Activity

For determination of antimicrobial activity, all the isolates were assayed against eight pathogenic test organisms: *Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Escherichia coli, Enterococcus faecalis, Salmonella typhi, Staphylococcus aureus and Klebsiella. pneumonia.*

It was observed that three isolates exhibited antibacterial activities against all the test organisms among four isolates. Among them isolate-1 and 2 showed satisfactory antimicrobial activity and isolate- 4 showed moderate or almost same activity and inhibited the growth of all the test organism. However, the highest antibacterial activity

of isolate-2 was exhibited against *E. coli* and highest antibacterial activity against *E. faecalis* was exhibited by isolate-1. Both of the isolates showed the lowest activity against *B. megaterium* and *S. aureus*. The isolate- 3 exhibited low level of antimicrobial activity and were unable to inhibit all the test organism.

Table- 3.9: Antimicrobial activity of all the isolates with zone of inhibition

Name of the test organism	Diameter of zone of inhibition in mm			
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**
Bacillus subtilis	17	18	0	14
Bacillus cereus	18	19	12	13
Bacillus megaterium	10	11	0	12
Escherichia coli	21	22	13	14
Enterococcus faecalis	18	17	12	11
Salmonella typhi	11	12	08	12
Staphylococcus aureus	12	11	0	11
Klebsiella pneumonia	16	15	0	09

Antimicrobial activities of the isolates are shown in the following figures:

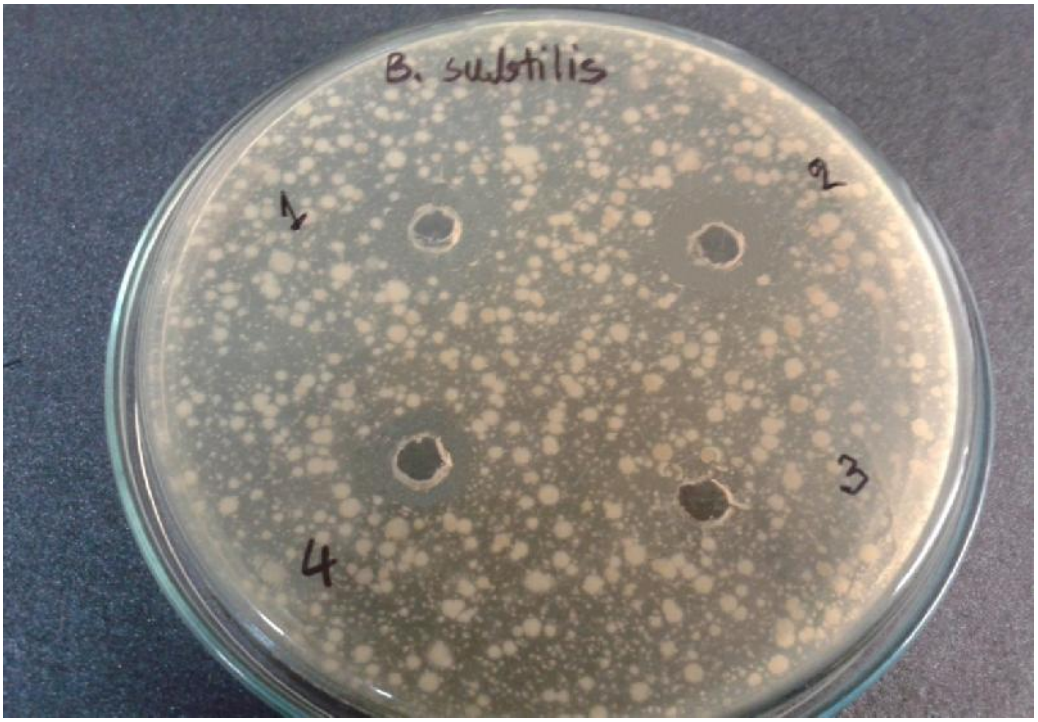

Plate-3.13: Antimicrobial activity of four isolates against *B. subtilis*

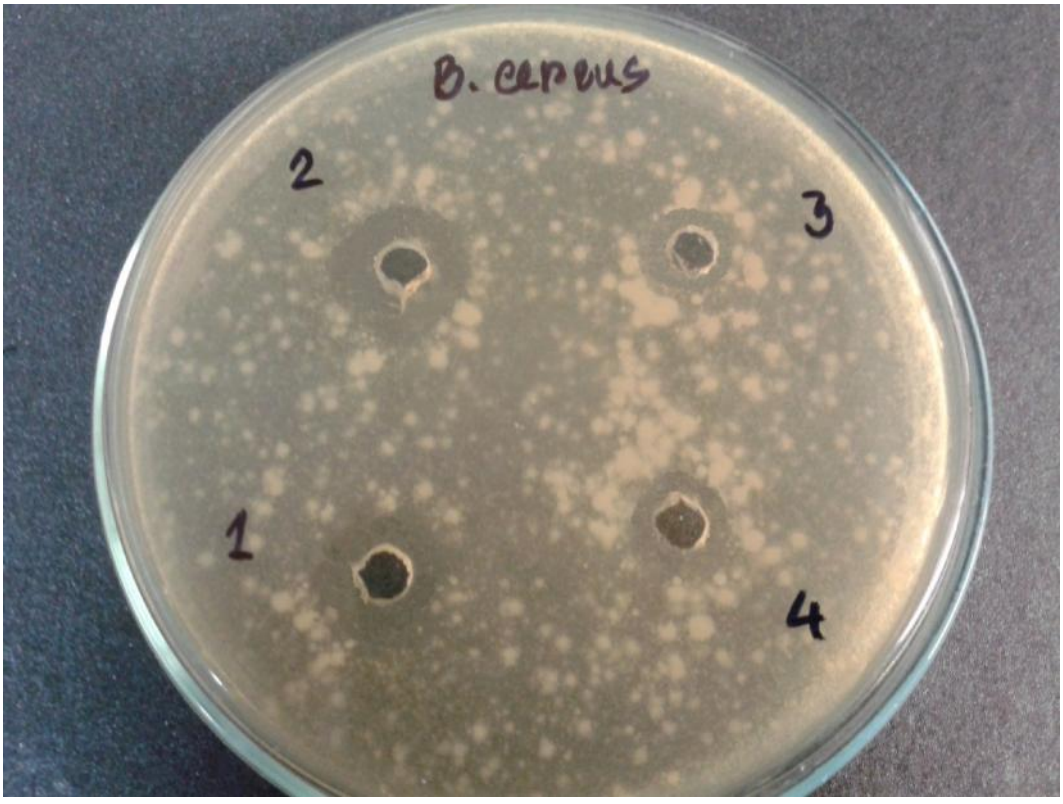

Plate-3.14: Antimicrobial activity of four isolates against *B. cereus*

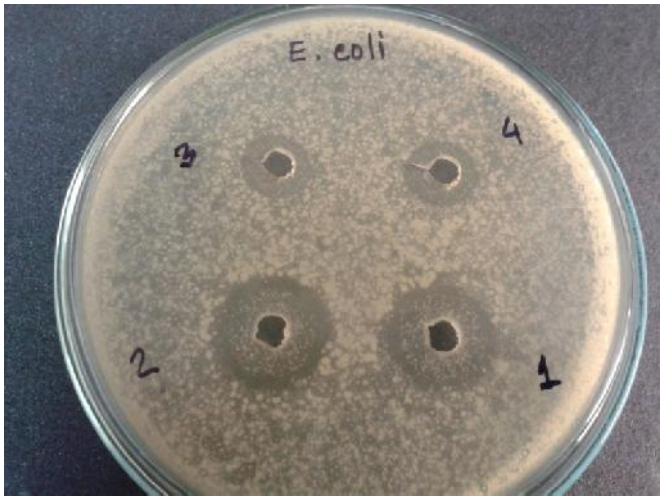

Plate-3.15: Antimicrobial activity of four isolates against *E. coli*

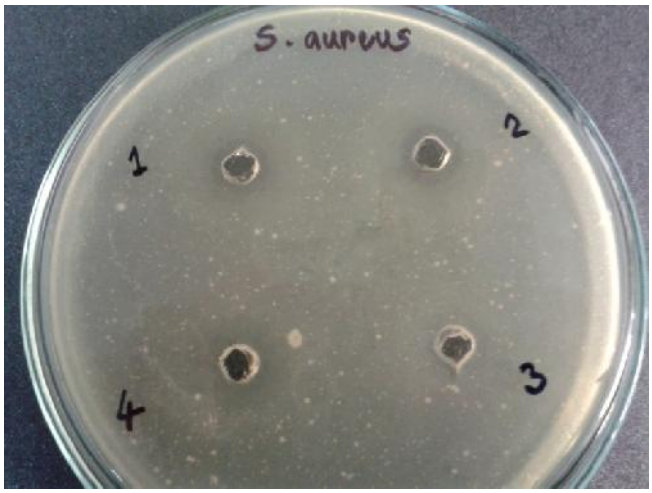

Plate-3.16: Antimicrobial activity of four isolates against *S. aureus*

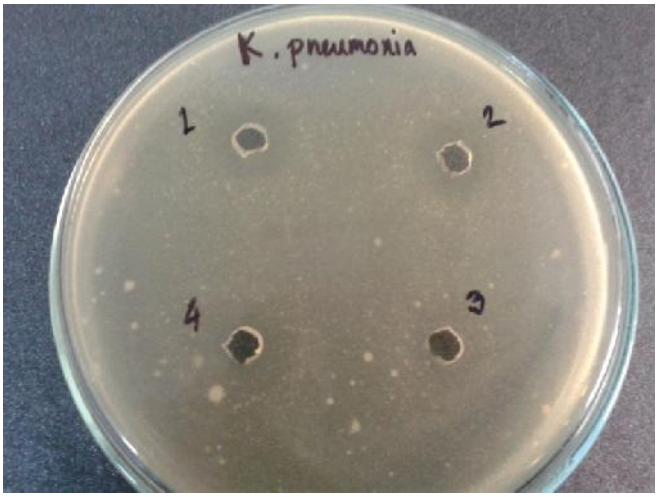

Plate-3.17: Antimicrobial activity of four isolates against *K. pneumonia*

5. DISCUSSION

About 100 trillion bacterial cells from at least 400 different species are found in the animal intestine, far exceeding the numbers of host cells & *Lactobacillus* is a dominant species that is found in intestine (Backhed *et al.* 2005). Lactic acid bacteria are demonstrated to be responsible for the spontaneous lactic acid fermentation of homemade dairy products, where three genera have previously been identified: *Lactobacillus*, *Enterococcus* and *Lactococcus* (Zambou *et al.* 2004). Zambou *et al.* (2008) demonstrated that strains of *Lactobacillus plantarum* constituted the dominant species of lactobacilli present in dairy products.

There is a lack of information about probiotic lactic acid bacteria in Bangladesh. The goal of this research work was to isolate potential probiotic bacteria & further select the most suitable strain of LAB from the shrimp for potential application as a probiotic supplement. Based on the morphological characteristics four (4) isolates were selected as probiotics from shrimp samples. After gram staining all the isolated microorganisms were identified as rod shaped, a convex, rough, smooth, shiny, irregular, circular, gram positive bacterium which indicates them to be member of *Lactobacillus sp.*

The isolates possess most of the characteristics of *Lactobacillus sp.* The significant growth of isolates on MRS agar plates in anaerobic conditions confirmed that isolates were *Lactobacillus sp.* IMViC test of selected isolates gave same results as *Lactobacillus sp*. The isolates were catalase negative that is a confirmation to be *Lactobacillus sp*. Another confirming test was the microorganisms did not interact with the artificial electron donor on oxidase application and concluded to be oxidase negative. Our present result of the IMViC, Catalase and oxidase test indicated that the isolated probiotic bacteria were *Lactobacillus sp*. All of the isolates were Indole, MR, VP, Citrate, Oxidase and Catalase negative & facultative hetero fermentative.

Lactobacilli have the most claims to be selected among potential probiotics (FAO/WHO 2002). Probiotics are health-promoting microorganisms. The criteria used to select potential probiotics are related to acid and bile tolerance, production of antimicrobial substances, cholesterol metabolism, production of useful enzymes and safety for food. *In vitro* survival of bacterial strains in low pH is a more accurate indication of the ability of strains to survive passage through the stomach. The organisms taken orally have to face

stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 (Corzo and Gilliland 1999).

In the present study, the spectrophotometer screening technique was used to test the ability of *Lactobacillus* strains to tolerate gastric acidic conditions. The results suggest that 4 selected isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there. Additionally, the tolerance to gastric transit was also observed to be variable among strains tested.

Kalui *et al.* (2009) demonstrated that the *L. plantarum* strains were tolerable to pH 2.5 after exposure for 3h and 10% of these strains could not at pH 2. Sirilun *et al.* (2010) reported that a viable rate of more than 90% of 43 out of 114 strains at pH 3 after 2h of incubation was found; at the pH 2 a surviving percentage that was higher than 50% could be observed in 27 strains. According to this statement, all isolates demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C.

All selected isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there. Many studies reported that tolerance to acid and other gastrointestinal stresses is strain specific (Morelli 2000; Huang and Adams 2004). For strains to survive and colonize the gastrointestinal tract, microorganisms should express tolerance to acid and bile salts (Gibson 1998). It has been suggested that food intake could protect bacteria during gastric passage (Charalampopoulos *et al.* 2002).

The pH, physical and chemical characteristics of a food carrier in which potential probiotics are relayed into the gut may have a buffering effect and significantly influence survival of the microorganisms (Patel *et al.* 2004) .This may also explain why *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*, known to exhibit poor survival when challenged *in vitro* to gastric acidity, showed high survival rates in the terminal ileum of fistulated minipigs fed with yoghurt (Lick *et al.* 2001).

Bile salt tolerance is the second selection criterion for probiotics. Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form in

volumes ranging from 500 to 700ml per day (Hoffman *et a.* 1983). The relevant physiological concentrations of human bile range from 0.1 to 0.3% (Dunne *et al.* 2001) and 0.5% (Mathara *et al.* 2008).

In present investigations, high bile salt tolerance was observed for all *Lactobacillus* strains tested. For a strain to be considered as probiotic, it should be able to survive at pH 3 and in the presence of 0.1% bile salt (Dunne *et al.* 2001). Thus, it is necessary that efficient probiotic bacteria should be able to grow in bile salt with concentration ranging from 0.15 - 0.30% (w/v) (Šuškovi *et al.* 2000). Kalui *et al.* (2009) reported that 18 of the 19 *L. plantarum* tested were able to grow in broth supplemented with 0.3% bile salts following exposure to pH 2.5. According to this statement, 4 out of the 16 lactobacilli tested in this study could be considered as potentially probiotic strains.

Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. It is believed that antibiotic used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora.

In present investigations, almost all tested isolates were sensitive to 7 of the 12 antibiotics used & all isolates showed resistance against Gentamicin & ciprofloxacin. Resistance of *Lactobacillus* against these antibiotics are good, as lactobacillus population will not decrease even after consumption of erythromycin and ciprofloxacin by humans to control gastroenteritis caused by enterobacteriacae and probiotic activity of *Lactobacillus* will not decrease and interfered by resistant antibiotics.

The antibiotic-resistant bacteria can transfer the resistance factor to other pathogenic bacteria through the exchange of genetic material (Mathur and Singh 2005). One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not contain transferable resistance genes. A recent study reported that the lactobacilli isolated from commercial products in Europe comprised strains resistant to tetracycline (29.5%), chloramphenicol (8.5%), and erythromycin (12%) and overall, more than 68% of the isolates exhibited resistance to two or more antibiotics (Temmerman *et al.* 2003).

Rojo-Bezares *et al.* (2006) reported that the most resistant species to the tested antibiotics were *L. plantarum* and *P. pentosaceus*; these authors also demonstrated that, *Lactobacillus* strains showed high minimum inhibitory concentration (MIC) values

(indicating high resistance) to ciprofloxacin [MIC of 50 to 64 μg/ml]. Similar results were previously reported by Elkins and Mullis (2004).

Glycopeptide, aminoglycoside and sulfamethoxazole resistance has been formerly described in LAB species (Mathur and Singh 2005) and in all cases it has been associated with their natural and intrinsic resistance, probably due to cell wall structure and membrane impermeability, complemented in some cases by potential efflux mechanisms (Elkins and Mullis 2004).

Liasi *et al.* (2009) demonstrated that, the 3 lactobacilli isolates tested (including one strain of *L. plantarum*) were susceptible to β-lactam group of antibiotic which include penicillin G, amoxicilline and ampicillin. The isolates were also susceptible to erythromycin, chloramphenicol and tetracycline. Furthermore, all isolates were also resistant to quinolones and sulfonamides. Herreros *et al.* (2005) demonstrated that in general, strains of lactobacilli showed significant resistance to ciprofloxacin and trimethoprim.

However, this could become threat as resistance mechanism could be plasmid mediated and can be conjugated to potential pathogens. Though the possibility of this is narrow, but chance still remains.

The antimicrobial activity is based on oxidative properties that result in irreversible changes in the microbial cell membrane. Many researchers and clinicians are interested in preventing or curing intestinal infections with probiotics, especially those caused by *E. coli*, *Shigella* sp., and *Salmonella* sp. The antibacterial activity of LAB is often due to the production of organic acids, diacetyl, hydrogen peroxide and bacteriocin or to bactericidal proteins formed during lactic acid fermentation.

The isolates from the gastrointestinal tract of shrimps were observed to have a higher inhibitory activity against *E. coli*, with a clear zone of 19 mm. It was observed that three isolates exhibited antibacterial activities against all the test organisms among four isolates. Among them isolate-1 and 2 showed satisfactory antimicrobial activity and isolate- 4 showed moderate or almost same activity and inhibited the growth of all test organism.

However, the highest antibacterial activity of isolate-2 was exhibited against *E. coli* and highest antibacterial activity against *E. faecalis* was exhibited by isolate-1. Both of the isolates showed the lowest activity against *B. megaterium* and *S. aureus*. The isolate- 3 exhibited low level of antimicrobial activity and were unable to inhibit all test organism.

6. CONCLUSION

The subtractive screening technique proposed in this study is a valuable tool for the characterization, identification, and selection of probiotic candidate strains, and could be used in large scale searches for probiotics. Lactic acid bacteria are able to inhibit growth of *E. coli* isolates, but with respect to yeasts, no such inhibitions were observed, and this is attributable to synergistic association between lactic acid bacteria and yeasts. The antimicrobial properties of *Lactobacillus* isolates were attributed mainly to the production of acid. Four strains were finally selected based on their desirable probiotic characteristics (resistance to bile and low pH, inhibition of intestinal pathogens, and attachment to intestinal cells) and lack of undesirable traits (harmful enzyme activity and acquired antibiotic resistance).

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APPENDIX i

Microbiological Media

Media used were prepared methods using appropriate compositions. Components used high grade and were produced either by sigma or Difco, USA. All media were sterilized by autoclaving for 15 minutes**.** The compositions used for different media have been shown below:

1. Simmons Citrate Agar

Ingredients	gm/l
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Sodium Ammonium Phosphate	1.0
Sodium Citrate,tribase	2.0
Sodium Chloride	5.0
Bromothymol Blue	.08
Agar	15.0

pH adjusted at(25°C)7.0±0.2

2. De Man, Rogosa and Sharpe (MRS) Broth

De man Rogosa and Sharpe (MRS)	gm/l
Pepton	10.0
Meat extract	10.0
Yeast extract	5.0
D(-)Glucose	20.0
Sodium acetate	5.0
Dipotassium hydrogen phosphate	0.2
Triammonium citrate	0.2
Magnesium sulfate heptahydrate	0. 05
Polysorbate 80	1.0

pH adjusted to 6.2-6.6 at 25 Final pH(at 25°C)7.4±0.2

HIMEDIA

Himedia Laboratories Pvt Ltd

3. MR-VP broth

Ingredients	Grams/Litre
Peptone from meat	7.0
Dextrose	5.0
Phosphate buffer	5.0

Final pH adjusted (at 25°C)

Reagents: Reagents which were used in carrying out different methods together with their sources are mentioned below:

1. Normal saline:

Ingredients	Amount
NaCl	8.5g

Distilled water	1.0 liter

pH was adjusted to 7.8

2. 5%Alcoholic alpha napthol solution

Ingredients	Amount
Ethanol	5g
Alpha napthol	100ml

3. KOH creatin solution

Ingredient	Amount
KOH	40g
Creation	300g
dH_2O	100ml

4. Methyl Red solution

Ingredient	Amount
Methy Red	100mg
Ethanol(95%)	300ml
dH_2O	200ml

5. Kovac's Reagent

Ingredient	Amount
Para-dimethyl amino benzaldehyde	5gm
Butyl Alcohol	75ml
Hcl	25ml

Appendix II

Common laboratory apparatus used in this research work

Instruments name	Model no.	Country
Autoclave	ALP	Japan
Autoclave	Model;Mc-40w,ALP Co. Ltd	Japan
Centrifuge machine	Mikro 120	Germany
Centrifuge machine	Sigma Z-16K	Germany
Centrifuge machine	Kubpta 6800	Japan
Electronic microscope	Olympus BX 41	Japan
Electrophoresis machine	ShimaDzu AUW22OD	Japan
Eyela natural oven	NDO-600ND,Tokyo Rikakikai C.l	Japan
Incubator(37°C)	Memmert	England
Incubator(25°C)	Wisecube	Korea
Oven	Memmert954,Schwabach	Germany
Hot water bath	Memmert	Germany
Laminar air flow	Holten	Japan
Magnetic Heating Stirrer	1100 hotplate &stirrer, Jenway	UK
Orbital shaker	GFL 3031	Germany
pH meter	Digital pH meter	Bangladesh
Refrigerator	General, ER-141F	Japan
Small weighting	AA-160, Dever Instrument CO	U.S.A
Spectrophotometer	Model T60U	
Transilluminator	AlphaInnotech	U.S.A
Peltier Thermal Cycler	BioRed	Mexico
Vortex-1	Whirlimixer, Fisons	England
Vortex-2	WiseMix	Korea
SDS-PAGE	Biorad	U.S.A