Volume-4, Issue-2, February-2020: 24-39 P-ISSN: 2659-1561
International Journal of Recent Innovations in Academic Research E-ISSN: 2635-3040

Research Article

Studies on Probiotic Properties of *Lactococcus lactis* Isolated From Indigenous Yogurt of Local Market in Bangladesh

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Received: Feb 3, 2020 **Accepted:** Feb 10, 2020 **Published:** Feb 14, 2020

Abstract: A total of 35 lactic acid bacteria were isolated from 8 different yogurt samples consumed in Bangladesh. Isolation were done using MRS agar and M17 agar plate incubated anaerobically at 37^0C for 72 hours. The isolates were identified with respect to colonial, cellular morphology, and biochemical characteristics. Among these isolates four of the strain was identified as *Lactococcus lactis* naming as AR, BF, BK and IG. Then these strains were inoculated for fermentation in different types of media in different pH such as 3.0, 4.0, 5.0 and 6.0. After fermentation period, the water extracts of fermented product were collected by centrifuge technique and tested for inhibitory activity against various pathogenic bacteria such as *Bacillus subtilis*, *Bacillus megaterium* and *Staphylococcus aureus* which is Gram positive bacteria and *Escherichia coli* and *Pseudomonas aeruginosa* which is Gram negative bacteria. Water extract compound showed antimicrobial effect against these organisms were determined by the agar disk diffusion method. The maximum inhibition zone on agar plate was observed in case of isolate-BF (Bonoful) (37.5 mm) against *Bacillus megaterium* while isolate-AR (Arong) have no inhibitory zone against Gram negative bacteria. Water extract of isolate-BK (Boishakhi) showed that, it inhibited the zone in higher rate (29.5 mm) in high concentration but lower rate (6.5 mm) in low concentration. The Minimum inhibitory concentrations (MIC) of isolate-BF extracted compound against *B. subtilis* and *E. coli* were 500 µgm/ml and 1000 µgm/ml respectively. The present study showed that *L. lactis* had a moderate antimicrobial property. In conclusion, most of the results from the present experiments showed that, there were variations in probiotic properties of the isolated *L. lactis* from Indigenous sources.
Keywords: Pathogenic bacteria, inhibition zone, *Lactococcus lactis.*

1. Introduction

In normal healthy animals, the gastrointestinal tracts are colonized by a complex microflora containing many different species. A balance of these microorganisms in the gastrointestinal tract is important not only in promoting efficient digestion and maximum absorption of nutrients, but also in increasing the capacity of the host in excluding infectious microorganisms and hence preventing disease (Walter *et al.,* 2003). *Lactobacilli* are common inhabitants of animal gastrointestinal tracts and many investigators have reported them to provide various positive health attributes. This group of "good" bacteria is generally called probiotics. Therefore, probiotics can be defined as living microorganisms that upon ingestion in certain numbers exert health effects beyond inherent basic nutrition (Gurner *et al*., 1998). Naidu *et al.*, (1999) describes probiotics as microbial dietary adjuvants that beneficially affect the hosts' physiology by modulating their mucosal and systemic immunity as well as improving the nutritional and microbial balance in their intestinal tracts. Probiotics are potentially useful in the management and treatment of various gastrointestinal diseases including diarrhea, inflammatory bowel disease, and colon cancer (Rolfe *et al.,* 2000).

Enhancement of nonimmunological gut defense barrier by probiotics may include maintenance of normal levels of intestinal permeability and micro-ecology, which is commonly shifted in the event of intestinal infection by pathogenic bacteria.

Vertebrate immune system can mount both innate and adaptive immune response in the event of infection by pathogenic microorganisms. Many types of immune cells are recruited to elicit an immune response and subsequently neutralize the pathogens. These cells include epithelial cells, natural killer cells, macrophages, neutrophils, dendritic cells and lymphocytes. These cells are quickly activated in the event of infection leading to production of an array of humoral mediators. Some may change their physiology and become phagocytic, yet others get involved in antibody synthesis and secretion. These activated immune cells may provide immediate protection against pathogens or promote specific immune responses. Therefore, these cells are useful in the probiotic enhancement of immunologic barrier in the animal gastrointestinal tract.

Probiotics have profound effects on potentiating both arms of immune responses. For instance, oral administration of the probiotic, *Bifidobacterium breve* was shown in mice that had been previously challenged with cholera toxin to promote humoral immunity by enhancing the secretion of immunoglobulin A (IgA) (Yasui *et al.,* 1999). In an investigation conducted by De Simone *et al.,* (1993), bacterial cell wall products were demonstrated to be able to not only enhance the proliferation of immune cells but also induce the expression of proinflammatory cytokines, which are necessary for the maintenance of a stable Th1/Th2 balance. This delicate balance is important for the host immune function as it dictates whether a humoral (antibody production) or a cell-mediated (cytotoxic T-cell) response should be mounted (De Simone *et al.,* 1993). Therefore, the influence of a probiotic strain of bacteria on the mammalian immune system can be easily evaluated through *in vitro* and *in vivo* measurement of cytokines, immunoglobulin production and lymphocyte proliferation.

The use of antibiotics is associated with the emergence of antibiotic-resistant bacteria, which have become difficult to control and have exerted adverse effects on the consumers of animal products. With the above positive attributes of probiotic bacteria on the prevention and disease management, probiotics hold great potential as a better alternative to antibiotics in farm animals.

Among lactic acid bacteria, *Lactococcus lactis* has extensively been employed in the food industry due to the preservative attributes of antimicrobial metabolites such as hydrogen peroxide, diacetyl, lactic acid and nisin (Lindgren and Dobrogosz, 1990). Nisin is a 34 amino acid peptide produced by certain strains of *Lactococcus lactis* which exhibits a broad spectrum of inhibitory activity against several Gram-positive bacteria of the genera Bacillus, Enterococcus, Listeria, Clostridium, and Staphylococcus (Tagg *et al.,* 1976; Chung *et al.,* 1989; Klaenhammer, 1993). This bacteriocin usually has little effect on Gram-negative bacteria, yeasts and moulds, although Gram-negative bacteria can be sensitised to nisin by permeabilization of the outer membrane by sublethal heating, freezing and chelating agents. (Delves-Broughton *et al.,* 1996). Besides, strains of *Lactococcus lactis* are frequently used on a large-scale as starter cultures in food industries for the manufacture of various fermented dairy products such as sour milk, cream, butter, fresh cheeses and many varieties of semi-hard cheeses (Prodelalova *et al.* 2005). In these applications, selected strains are used as food supplements that may favorably influence the intestinal flora of human and animal hosts, such as competitive exclusion of gastrointestinal pathogens, stimulation of immune responses or antimutagenic and anticancerogenic activities (Mercenier *et al.,* 2003; Reid *et al.,* 2003).

Because of their long-time use in various food and feed preparations, *Lactococcus lactis* has been given the so-called GRAS (generally recognized as safe) status (Federal Register, 1988). This indicates that *Lactococcus lactis* strains are food-grade organisms without imposing a health risk for the consumers or the environment. However, by the presence and expression of virulence genes and/or antibiotic resistance genes in food-associated LAB such as *Lactococcus lactis* strains on mobile genetic elements such as plasmids or (conjugative) transposons, these antibiotic resistance traits can potentially be transferred to the human or animal commensal flora and to pathogenic bacteria which are temporarily residing in the body of hosts and provide a survival benefit to the invading microorganisms. Under such circumstances it is difficult to eliminate the infection from the host body caused by pathogens.

Therefore, the fermentative and nutritional LAB such as *Lactococcus lactis* that is consumed on a daily basis worldwide must be characterised to ensure the absence of acquired antimicrobial resistance properties so that these would be safe for human and animal consumption (Belen *et al.,* 2005; Liasi *et al.,* 2009). Therefore, to contribute to the potential uses of *Lactococcus lactis isolates* in the food and dairy industries, the isolation, identification, antimicrobial activity, and antibiotic susceptibility determination of four strains of *Lactococcus lactis* from yogurt have been investigate.

2. Materials and Methods

2.1 Site of sample collection

A total of eight (08) yoghurt samples were collected from local markets of Dhaka, Tangail and Narayangonj Districts of Bangladesh due to their wide acceptance among the consumers of Bangladesh. The samples were collected in sterile universal tubes and kept cool until they could be taken to the laboratory, where they were kept at 4ºC for further use.

2.2 Isolation of lactic acid bacteria

The samples were aseptically weighted and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making fold serial dilution. 0.1 ml from each dilution was then sub cultured, in duplicate, into the M17 and MRS agars (Merck, Germany) used for isolating LAB (Badis *et al*., 2004a). To prevent the growing of yeasts, the media were then supplemented with 100 mg/L of cycloheximide before being incubated at the appropriate temperatures (42ºC, 35ºC and 30ºC) for 2-3 days (Beukes *et al*., 2001; Kalavrouzioti, 2005). To perform the total counts, the higher dilutions were used. Colonies were randomly selected and streak plating was then used to purify the strains which were subsequently kept in two different conditions including at 4°C for MRS and plates and at -20°C for M17 and MRS supplemented by 20% glycerol for further use (Mathara *et al*., 2004).

2.3 The preparation of medium and inoculation

Using semi-solid N-free bromothymol blue (Nfb) medium (Dobereiner, 1980) with malate as the carbon source, it became very easy to enrich and isolate *Azospirillum* from the soil and roots. The pH of the medium was adjusted to 6.8 (green colour) just prior to adding agar. 7 ml of the medium was taken in each 15 ml screw-capped test tube and sterilized by autoclaving. Nfb semi-solid medium in screw-capped tubes was inoculated with 0.1 ml of each sample suspension using a sterile pipette.

2.4 Incubation and observation

The tubes were incubated at 37°C for 72 hours. After incubation *Azospirillum* appeared in the tubes forming characteristic thin dense, white pellicle few mm below the surface at the

medium (Dobereiner, 1980). The pellicles were examined microscopically for the presence of gram negative, vibroid and actively motile cells.

2.5 Biochemical confirmation of the bacterial strain

Biochemical tests like Motility test, indole test, methyl red test, voges proskeur's test, citrate utilization test, oxidase test, catalase test, TSI agar were performed for all the strains.

2.5.1 Motility test

This test was done to determine the motility of bacteria. Tubes containing 7 ml of motility test agar in duplication were inoculated by stabbing through center of the medium with inoculating needle to approximately one-half the depth of the medium. 24 hours old cultures of the selected strains were used for inoculation. The tubes were incubated at 37°C for 48 hours. Development of diffuse growth spreading from the line of inoculation or diffuse growth throughout the entire medium or diffusion from one or two points only, appearing as nodular growth along the stab line indicated the positive result.

2.5.2 Oxidase test

Oxidase enzyme of bacteria is capable of transferring electrons to a substance tetra-p-phenylenediamine dihydrochloride, which becomes purple in color. For this test a piece of autoclaved filter paper was placed in a clean Petri dish and 2-3 drops of freshly prepared oxidase reagent was added. A colony of the organism was removed by using a glass rod and it was smeared on the filter paper. The development of blue purple color within few seconds (10 seconds) indicated oxidase positive.

2.5.3 Catalase reaction

The enzyme catalase is capable of decomposing hydrogen peroxide into water and molecular hydrogen. For the test, nutrient broth tubes were inoculated with 24 hr. old cultures of selected strains with the help of pasteur pipettes and incubated at 37°C for 72 hr. After incubation, 1 ml of 3% hydrogen peroxide solution was added to each of the tubes. The production of bubbles indicated the positive result.

$H_2O_2 \rightarrow H_2O + H_2\uparrow$

2.5.4 Nitrate reduction test

Capability of the organism to carry out reduction of nitrate to nitrite can be determined by this test. The formation of nitrite indicated the presence of the enzyme nitrate reductase in the organism. The tubes of nitrate broth in duplicates were inoculated with 24 hours old cultures of selected strains and then incubated at 37°C for 72 hours. After incubation a few drops (equal volume) of solution A and B were added and the tubes were shaken well. The formation of red color indicated the reduction of nitrate to nitrite.

2.5.5 Hydrolysis of starch

Starch agar plates were inoculated with 24 hr. old selected strains by streak method and the plates were incubated at 37°C for 72 hr. After growth, iodine solution was added to each of the plates. Development of blue color indicated that starch had not been hydrolyzed. Complete hydrolysis of starch would be indicated by the development of clear white and bluish or brownish white color respectively.

2.5.6 Citrate Utilization

This test was done to determine the ability of bacteria to utilize citrate as sole source of carbon for metabolism. Simmon's citrate agar slants were inoculated with 24-48 hours old

cultures of the test organism and incubated at 37°C temperature for 24 to 48 hours. The color change of the medium from green to blue indicates a positive result.

2.5.7 Fermentation test

Fermentation test is of considerable significance test for the identification and classification of bacteria. The microorganisms differ in their ability to ferment different carbohydrates. Some of them upon fermentation of carbohydrates produce both acid and gas; the others produce only acid and no gas. There are still other which can not ferment carbohydrates at all. In this study the fermentation test of the following carbohydrates and sugars were done. Fermentation tubes with fermentation broth medium were made using bromothymol blue as indicator (One durham tube was introduced in each of the fermentation tubes).

Inoculation with 24 hr. old culture of selected strains were used and then incubated at 37°C for 72 hr. The change of color of the indicator yellow showed the production of acid. Blue color of solution indicated alkaline. If CO_2 formed during fermentation it would be collected in the durham tubes.

Table 2.1. Different carbohydrate sources.

Monosaccharides	Pentose: Arabinose, Rhamnose, Ribose, D-xylose
	Hexose: D-glucose, Fructose, Galactose, Cellobiose
Disaccharides	Sucrose, Lactose, Maltose
Sugar alcohol	Mannitol, Inositol, Dulcitol

2.5.8 Freeze-drying

Pure cultures of all strains were transferred from frozen stock cultures into MRS broth and incubated at 37^0C for 24 hour and three successive transfers were carried out. The cultures were then centrifuged at 4500 × g at 4^0C for 15 min. The cell pellet was suspended in twenty milliliters of sterile 0.1M sodium phosphate buffer (pH 6.8) and re centrifuged (2714×g at 4^0C, 15 min). The cell pellet was suspended in sodium phosphate buffer (20 mL) at 2% (wt/vol) as a cryoprotectant. The cell suspension was then aseptically poured into large petri dishes, sealed with paraffin and aluminum foil, and frozen overnight at -20^0C, followed by freeze-drying for 48 hr using a freeze-dryer. The freeze dryer was programmed to operate for 10 min of initial freezing after internal pressure was reduced to -100 Torr, 44 hr of primary freezing, and 4 hr of secondary freezing.

The temperature was maintained at -65^0C. Freeze-dried cells were stored in sterile, sealed, plastic bags. An initial bacterial count was performed using MRS agar at 37^0C for 48 h. They were then stored separately in sealed plastic bags in a freezer at -20^0C, and at room temperature (approximately 20^0C) for enumeration of viable population and proteolytic activity.

2.6 Fermentation media

2.6.1 MRS broth and M17 agar

Lactobacilli MRS broth and Lactobacilli M17 agar are recommended for use in the isolation, enumeration and cultivation of *Lactobacillus* and some *of Lactococcus* species. M17 Agar and MRS Broth contain peptone and dextrose. These ingredients supply nitrogen, carbon and other elements necessary for growth. Polysorbate 80, acetate, magnesium and manganese provide growth factors for culturing a variety of lactobacilli. The above ingredients may inhibit the growth of some organisms other than lactobacilli.

2.6.2 Corn step liquor (CSL)

Corn steep liquor is a by-product of corn wet-milling (Liggett and Koffler, 1948). A viscous concentrate of corn solubles which contains amino acids, vitamins minerals and organic nitrogen, and also an important constituent of some growth media. It was used in the culturing of *Penicillium* during research into penicillin by American microbiologist Andrew J. Moyer (Liggett and Koffler, 1948).

2.7 Antimicrobial activity of water extract of *Lactococcus lactis* fermentation (Disk Diffusion Tests)

Disc diffusion method was used to test the antimicrobial activity of PCA. The direct colony suspension method which is the most convenient method was used for inoculum preparation. Using a sterile inoculating loop, four or five isolated colony of the organism to be tested was touched from the subculture plate. The organism was suspended in 5 ml of nutrient broth. The broth containing test tube was vortexed to create a smooth suspension.

2.7.1 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, a 0.5 McFarland standard solution was used. This results a suspension containing approximately 1 to 2×10^8 colony forming units (CFU)/mL.

2.7.2 Determination of minimal inhibitory concentration (MIC)

MIC was defined as the lowest concentration of antimicrobial agent that inhibited bacterial growth, as indicated by the absence of turbidity. MICs values were determined by the micro titer broth method (Amsterdam *et al*, 1996) in sterile flat-bottom 96-well polystyrene plates. Serial dilution techniques were used to determine the MICs of plant extracts and antibiotics.

Isolated colonies were aseptically inoculated in sterile nutrient broth and vortex and then turbidity of inoculum was adjusted to 0.5 McFarland using 0.9% (w/v) sterile normal saline. 100 times diluted inoculum from this inoculated nutrient broth was used. A final concentration of 10^5 cfu/ml of tested bacteria was added to each dilution.

3. Results

3.1 Isolation of *Lactococcus lactis* from selective media

Lactococcus lactis were isolated from selective M17 agar media. 4 (Four) isolates obtained were morphologically characterized according to their colony characteristics along with their Gram reaction and microscopic examination. Only Gram positive, non-motile, spherical-shaped bacteria, showing phenotypic characters like *Lactococcus* species on M17 agar media were selected for further experiments.

The basis for selection was that these isolates could probably belong to the Genus *Lactococcus*, which has been shown to have probiotic attributes (Ljungh *et al.,* 2006).

3.2 Identification of isolates

Bacteria isolated from different yogurt were identified as *Lactococcus lactis* by observing some physiological & biochemical test. They are spherical-shaped, gram-positive cocci and short chains. The bacterium is non-sporulating, mesophilic and non-motile. When cultured in the lab on nutrient agar, it appears bright orange color. The isolates could coagulate milk and were able to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and showed growth in MRS broth containing 1-9% NaCl. All these key features helped to classify the isolates as lactococci

Table 3.1. Morphological and physiological characteristics of the selected strain BF.

Tests	Isolate-1 BF	Isolate-2 BK	Isolate-3 IG	Isolate-4 AR
Gram staining	Positive	Positive	Positive	Positive
Endospores	Negative	Negative	Negative	Negative
Motility	Negative	Negative	Negative	Negative
Oxidase test	Negative	Negative	Negative	Negative
Catalase test	Negative	Negative	Negative	Negative
Urease test	Negative	Negative	Negative	Negative
Nitrate reduction	Reduced	Reduced	Reduced	Reduced
Starch hydrolysis	Negative	Negative	Negative	Negative
Citrate utilization test	Negative	Negative	Negative	Negative
Fermentation test				
a) Glucose	Positive	Positive	Positive	Positive
b) Sucrose	Positive	Positive	Positive	Positive
c) Mannitol	Negative	Negative	Negative	Negative
d) Lactose	Positive	Positive	Positive	Positive
e) Fructose	Negative	Negative	Negative	Negative
f) Raffinose	Positive	Positive	Positive	Positive
g) Galactose	Negative	Negative	Negative	Negative
h) Xylose	Negative	Negative	Negative	Negative
i) Mannose	Positive	Positive	Positive	Positive
Glucose as sole carbon source	Negative	Negative	Negative	Negative
Sucrose as sole carbon source	Negative	Negative	Negative	Negative

Identification

The Four (4) strains were identified and designated as:

1) *Lactococcus lactis* (BF)
2) *Lactococcus lactis* (BK)
3) *Lactococcus lactis* (IG)
4) *Lactococcus lactis* (AR)

BF-Bonoful; BK-Boishakhi; IG-Igloo; AR-Arong

Taxonomical position of *Lactococcus lactis*

Kingdom: Bacteria
Division: Firmicutes
Class: Bacilli
Order: Lactobacillales
Family: Streptococcaceae
Genus: Lactococcus
Species: *L. lactis*

3.3 Antibiotic susceptibility test for specific pathogenic bacteria

Total four isolates of bacteria were selected for antibiotic susceptibility test. Sixteen antibiotics were analyzed for each type of bacteria to see the sensitivity and resistance toward antibiotics. In this study eight isolates individually were tested one time for sixteen types of antibiotics. Two tables are prepared according Gram negative and Gram positive bacteria and showed sensitivity, resistance and intermediate according to the clear zone diameter. Some bacteria have shown no clear zone which means it was resistant to the specific antibiotic and some shown very small diameter of clear zone this also an indicator of anti-biotic resistance. If clear zone diameter is larger than resistant diameter scale and less than susceptible diameter than this result called intermediate, which means the specific bacteria is neither resistant nor susceptible to the specific antibiotic.

The clear zone diameter scale when matches the susceptibility diameter scale then the bacteria is sensitive that specific antibiotic. The standard diameter scale is organized as a chart by antibiotic manufacturing companies and the test outcome is then matched with this chart to interpret the result.

Table 3.2. Zone inhibition (diameter) of samples against *Escherichia coli* compared with Ciprofloxacin (CIP) standard disk (15 µgm/disk)

Plate No.	Inhibited zone diameter (mm)				
	AR	BK	IG	BF	**CIP**
1 (512µgm/disk)	8	36.5	28.5	29.5	35
2 (256µgm/disk)	No inhibition	29.5	28.5	22.5	33.5

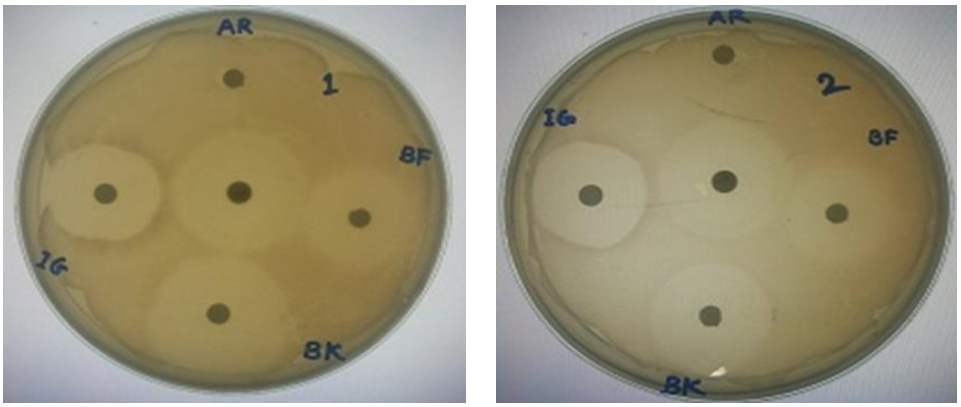


Plate 1 (10 times diluted) **Plate 2 (20 times diluted)**

Figure 3.1. Zone inhibition (diameter) of samples against *Escherichia coli* compared with Ciprofloxacin (CIP) standard disk (15 µgm/disk)

Table 3.3. Zone inhibition (diameter) of samples against *Pseudomonas aeruginosa* compared with Ceftazidime (CAZ) standard disk (15 µgm/disk)

Plate No.	Inhibited zone diameter (mm)				
	AR	BK	IG	BF	**CAZ**
3 (512µgm/disk)	No inhibition	29.5	12	25.5	25
4 (256µgm/disk)	No inhibition	6.5	12	22.5	25.5

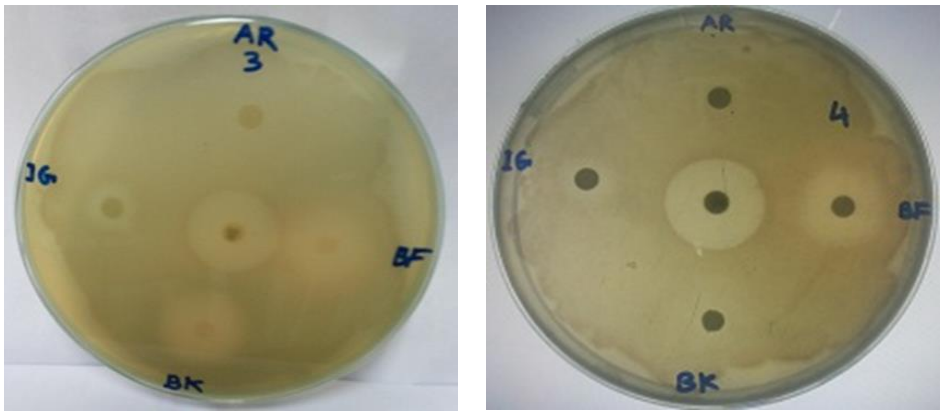


Plate 3 (10 times diluted) **Plate 4 (20 times diluted)**

Figure 3.2. Zone inhibition (diameter) of samples against *Pseudomonas aeruginosa* compared with Ceftazidime (CAZ) standard disk (15 µgm/ disk)

Table 3.4. Zone inhibition (diameter) of samples against *Staphylococcus aureus* compared with Erythromycin (EAR) standard disk (10 µgm/ disk)

Plate No.	Inhibited zone diameter (mm)				
	AR	BK	IG	BF	**EAR**
5 (512µgm/disk)	15.5	18.5	No inhibition	12	12.5
6 (256µgm/disk)	13.5	7.5	No inhibition	12	12.5

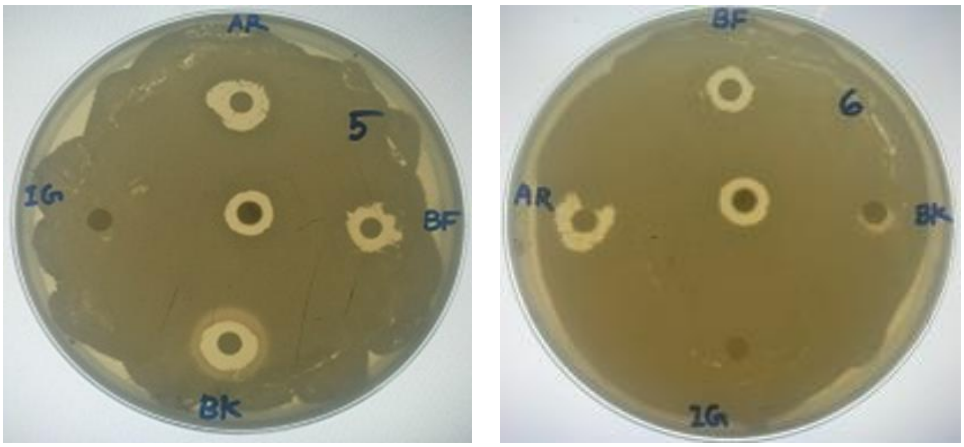


Plate 5 (10 times diluted) **Plate 6 (20 times diluted)**

Figure 3.3. Zone inhibition (diameter) of samples against *Staphylococcus aureus* compared with Erythromycin (EAR) standard disk (10 µgm/ disk)

Table 3.5. Zone inhibition (diameter) of samples against *Bacillus subtilis* compared with Co-trimoxazole (SXT) standard disk (25 µgm/ disk)

Plate No.	Inhibited zone diameter (mm)				
	AR	BK	IG	BF	**SXT**
7 (512µgm/disk)	29	25	23	34.5	34.5
8 (256µgm/disk)	28.5	18	22	29.5	33.5

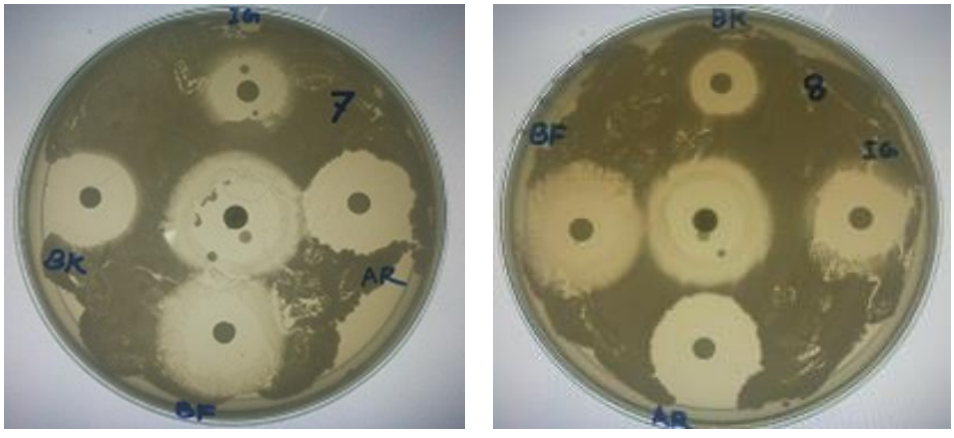


Plate 7 (10 times diluted) **Plate 8 (20 times diluted)**

Figure 3.4. Zone inhibition (diameter) of samples against *Bacillus subtilis* compared with Co-trimoxazole (SXT) standard disk (25 µgm/ disk)

Table 3.6. Zone inhibition (diameter) of samples against *Bacillus megaterium* compared with Ampiclin (AMP) standard disk (25 µgm/ disk)

Plate No.	Inhibited zone diameter (mm)				
	AR	BK	IG	BF	AMP
9 (512µgm/disk)	17	18.5	8	37.5	13
10 (256µgm/disk)	9.5	14	8	33.5	9.5

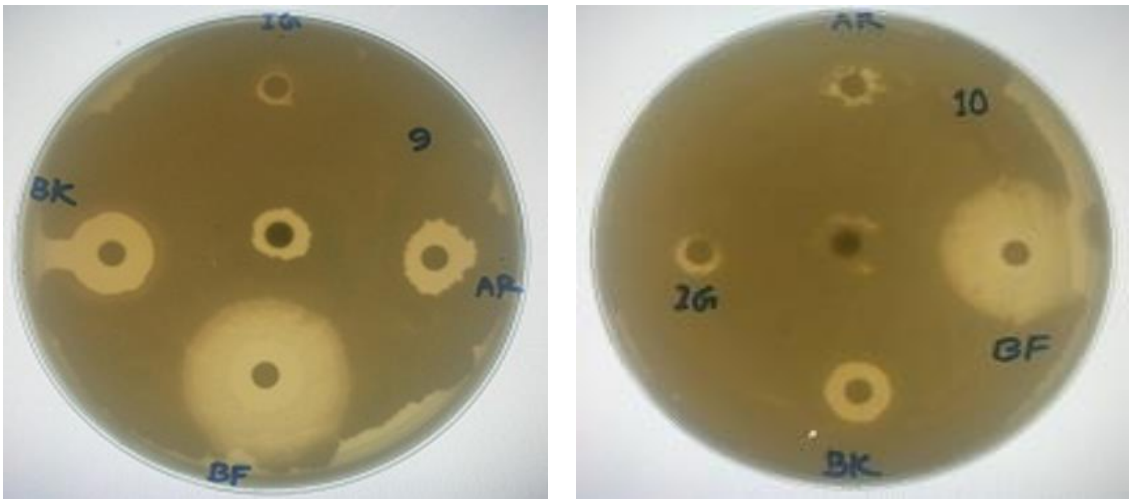
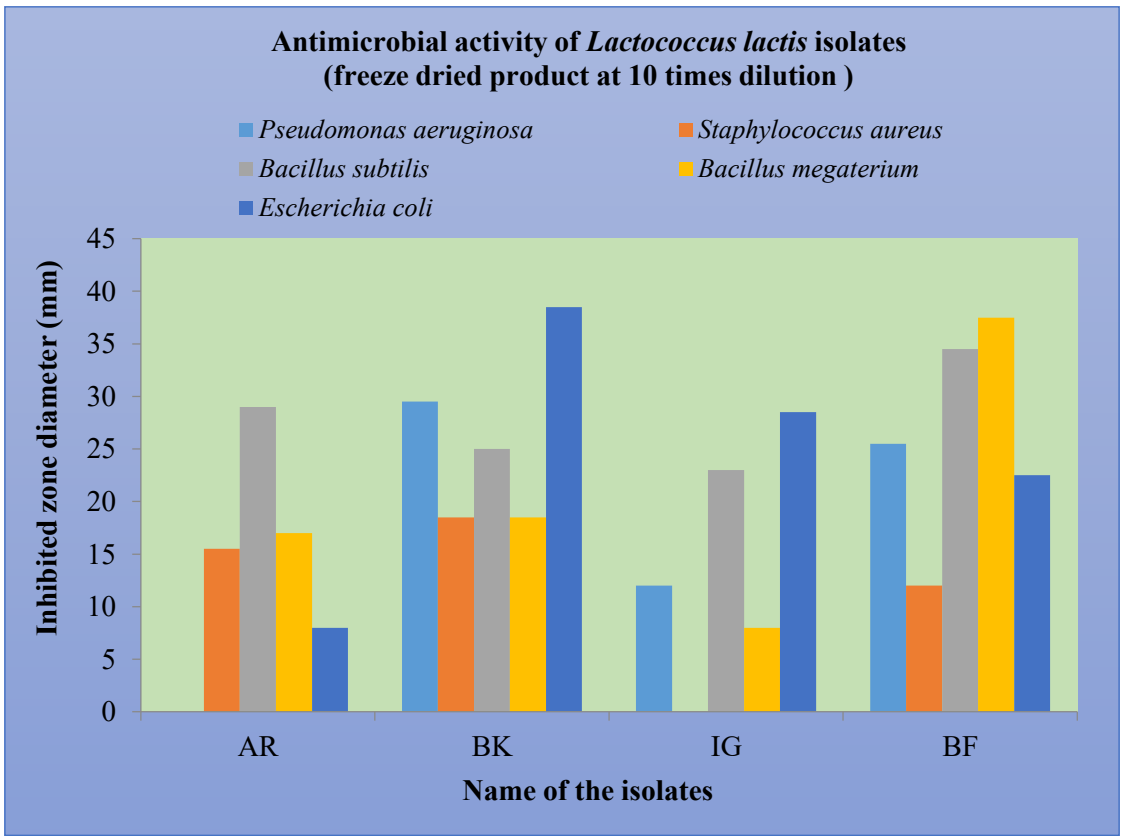


Plate 9 (10 times diluted) **Plate 10 (20 times diluted)**

Figure 3.5. Zone inhibition (diameter) of samples against *Bacillus megaterium* compared with Ampiclin (AMP) standard disk (25 µgm/ disk)



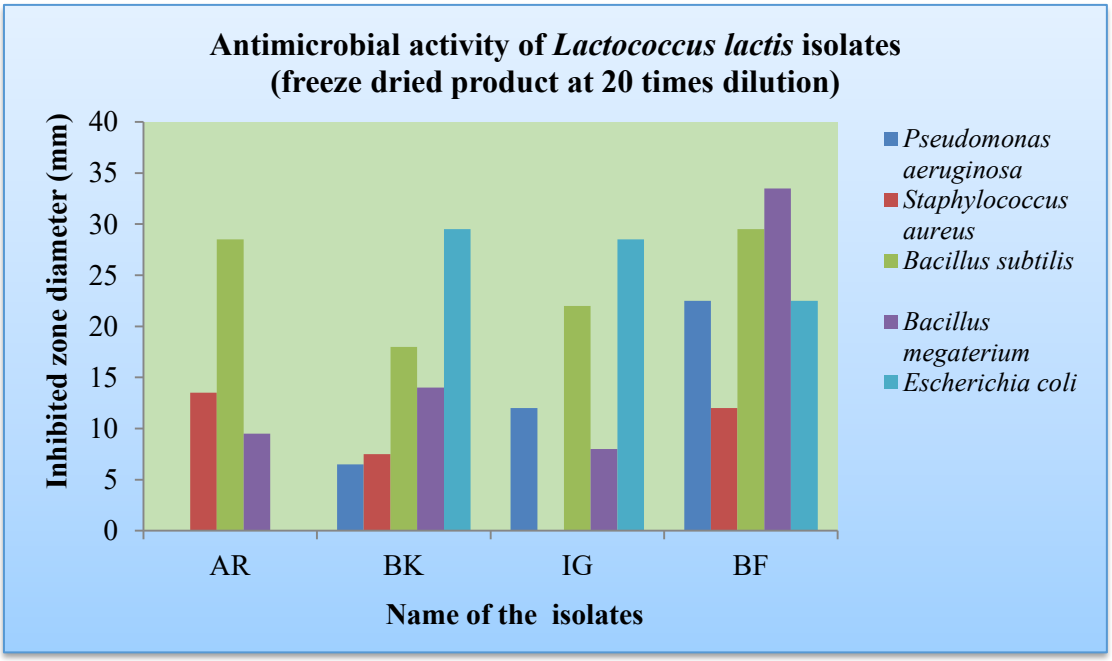


Figure 3.6. Antimicrobial activity of *Lactococcus lactis* isolates (freeze dried product at 10 times and 20 times dilution)

3.4 Minimum inhibitory concentration (MIC) of the water extract

The minimum inhibitory concentration (MIC) of the crude chloroform extract was determined against *Escherichia coli and Bacillus subtilis by serial dilution method.* The concentrations at which first sign of inhibition observed in the experiment against respective test organisms were 1000 and 500 µgm/ml respectively. No inhibition was observed in test tubes containing compound lower than the above-mentioned concentrations against respective test organisms. Growth of the organism was observed in the test tube C_I (Medium + Inoculum) and growth was observed in test tube C_M (Medium) and C_S (Medium + Sample). The results are shown in the following tables.

Table 3.7. MIC of the water extract against *Escherichia coli* for isolate-BF

Test tube number	Water extract (µgm/ml)	Observation **against *Escherichia coli***	Observation **against *Bacillus subtilis***
1	1000	N.G.	N.G.
2	750	N.G.	N.G.
3	500	G	N.G.
4	250	G	G
5	100	G	G
6	50	G	G
7	25	G	G
8	12.5	G	G
C_S	1000	N.G.	N.G.
C_I	0	G.	G.
C_M	0	N.G.	N.G.

*Cs-Medium + Sample, CI-Medium + Inoculum, CM-Medium, G-growth, N.G-No growth

The MIC of the compound is 1000 µgm/ml and 500 µgm/ml against *Escherichia coli* and *Bacillus subtilis* for isolate BF respectively.

Figure 3.7. MIC of the water extract against *Escherichia coli* and *Bacillus subtilis* for isolate BF

4. Discussion

The interest in *Lactococcus lactis* has been due to its application as starter culture for yoghurt and soft cheese making as well as its probiotic use (Dal Belo *et al.,* 2012). *L. lactis* is used as a probiotic bacterium because it was shown to improve lactose digestion and absorption in lactose intolerant people. It also has more potential and safety in developing vaccine in human (Bello *et al.,* 2012.). In this study, *L. lactis* was isolated from indigenous yoghurt and identified by several biochemical techniques (Chenbey *et al.,* 2000). Two types of media were chosen for the isolation of LAB. First, MRS was used as a medium for LAB which gave a general scope of the flora present in the samples. M17 agar was used as a selective medium for the isolation of *L. lactis.* It was aimed to select a bacteriocin producer strain of *L. lactis* and could be used as probiotic bacterium by its ability to antagonize pathogens in human gut. The antibacterial substance produced by *L. lactis* inhibited the growth of some sensitive Gram (+) positive and Gram (-) negative bacteria including some food-borne pathogens. Consequently, it has been coupled with most of the definitions of bacteriocins (Klaenhammer, 1988). The screening of LAB isolates for antibacterial activity revealed that *L. lactis* isolates were able to produce some inhibitory compound against the spoilage causing and pathogenic bacteria.

Two *Lactococcus* isolates naming BF & BK giving broad inhibition zone were selected for further evaluating the effectiveness of antibacterial activity of the extracted bacteriocins.

In this study it was found that the inhibitory activity of bacteriocin producing *L. lactis* isolates revealed that the maximum inhibition zone was observed in case of isolate BF (37.5 mm) against *Bacillus megaterium* while least was observed in isolate AR (No inhibition). The water extract of isolate AR fermentation has no significant inhibitory effect on Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* compare with the standard antibiotic disk, but they have inhibitory effect on several Gram positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus megaterium.*

Moreover, the water extract of BK fermentation showed that its antimicrobial activity was significantly increased for both Gram positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium* and Gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa*. It suggests that higher concentrated dose have higher inhibitory effect on bacteria. But water extract of isolate IG fermentation showed a very different result from BK. IG showed that if the concentration was increased there were no significant change on zone inhibition. Higher concentration and lower concentration showed approximately similar result against specific bacteria for isolate IG (Table no: 3.2, 3.3, and 3.5).

In case of isolate-BF it was found that, the water extract of BF fermentation product showed more inhibitory effect on Gram positive bacteria than Gram negative bacteria. It suggests that the *L. lactis* shows more antimicrobial properties against the Gram positive bacteria than Gram negative bacteria (Table no: 3.2 and 3.5).

From this study, it is clear that, the water extract from all the isolates have significant antimicrobial activities against all the test organism irrespective to Gram positive or Gram-negative bacteria.

The Minimum inhibitory concentrations (MIC) of the water extract of isolate BF fermentative product were found at moderate concentration for both Gram positive and Gram negative bacteria by serial dilution method. The concentrations at which first sign of inhibition observed in the experiment against respective test organism indicated that the water extract of BF fermentation product has a moderate antimicrobial property.

MIC of the water extract against *Escherichia coli* for isolate BF was 750 µgm/ml and against *Bacillus subtilis* was 500 µgm/ml. MIC results also suggests that water extract of fermentative product from BF has more inhibitory effect on Gram positive bacteria then that of Gram-negative bacteria.

The inhibitory action of *L lactis* bacteria can be due to the accumulation of main primary metabolites such as lactic and acetic acids, ethanol and carbon dioxide. Additionally, *L. lactis* are also capable of producing antimicrobial compounds such as hydrogen peroxide, diacetyl, acetoin and bacteriocins such as nisin. The production levels and the proportions among those compounds depend on the strain, medium compounds and physical parameters.

The inhibitory activities of *L. lactis* against Gram positive pathogens have been mostly shown to be due to the bactericidal effect of protease sensitive bacteriocins. However, the antagonistic effects of LAB towards Gram negative pathogens could be related to the production of organic acids and hydrogen peroxide.

5. Conclusion

In conclusion, the results obtained from this study demonstrated the remarkable antimicrobial attributes of the isolated *L. lactis* species from yogurt. According to previous studies, a large number of lactic acid bacteria strains with different bioactive potentials especially in the form of antimicrobial properties have been identified from a variety of fermented product sources these scientific evidences have been a motivating factor to choose a yogurt based fermented product which could further confirm the results of this study.

On the other hand, such positive outcomes would be a leading point towards application of simple worthy traditional methods such as fermentation in producing natural healthy food products and encouraging consumers to include such valuable food items into their eating habits. Hope these friendly food groups would be added to daily diet of everyone to improve their body immunity hence, decreasing unnecessary intake of chemical antibiotics. However, further in vitro and in vivo studies are required according to selection criteria including adhesion to mucosal cells of the gastrointestinal tract, bile salt and acid tolerance, bile salt hydrolase activity, viability, resistance to antibiotics, safety and organoleptic properties to be applicable in different food products such as starter culture in fermented dairy products.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Badis, A., Guetarni, D., Moussa-Boudjemaa, B., Henni, D.E., Tornadijo, M.E. and Kihal, M. 2004. Identification of cultivable lactic acid bacteria isolated from Algerian raw goat's milk and evaluation of their technological properties. Food Microbiology, 21(3): 343-349.
2. Chèneby, D., Philippot, L., Hartmann, A., Hénault, C. and Germon, J.C. 2000. 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. FEMS Microbiology Ecology, 34(2): 121-128.
3. Chung, K.T., Dickson, J.S. and Crouse, J.D. 1989. Effects of nisin on growth of bacteria attached to meat. Applied and Environmental Microbiology, 55(6): 1329-1333.
4. Dal Bello, B., Cocolin, L., Zeppa, G., Field, D., Cotter, P.D. and Hill, C. 2012. Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese. International Journal of Food Microbiology, 153(1-2): 58-65.
5. Davidson, P.M. and Branen, A.L. pp. 441-468. Marcel Dekker Inc., New York.
6. Davidson, P.M., Post, L.S., Braner, A.L. and McCurdy, A.R. 1983. Naturally occurring and miscellaneous food antimicrobials. In: Antimicrobials in Foods. (Eds.), Davidson, P.M. and Branen, A.L. pp. 385-392. Marcel Dekker Inc., New York.
7. De Simone, C., Vesely, R., Bianchi Salvadori, B. and Jirillo, E. 1993. The role of probiotics in modulation of the immune system in man and in animals. International Journal of Immunotherapy, 9(1): 23-28.
8. Delves-Broughton, J., Blackburn, P., Evans, R.J. and Hugenholtz, J. 1996. Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek. 69: 193–202.
9. Federal, R. 1988. Nisin preparation affirmation of GRAS status as a direct human food ingredient. Food Registration, 54: 11247-11251.

10. Flórez, A.B., Delgado, S. and Mayo, B. 2005. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Canadian Journal of Microbiology, 51(1): 51-58.

11. Gurner, F. and Schaafsma, G.J. 1998. Probiotics. International Journal of Food Microbiology, 237-238.

12. Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochemie, 70(3): 337–349.

13. Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Reviews, 12(1-3): 39-85.

14. Liasi, S.A., Azmi, T.I., Hassan, M.D., Shuhaimi, M., Rosfarizan, M. and Ariff, A.B. 2009. Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. Malaysian Journal of Microbiology, 5(1): 33-37.

15. Liggett, R.W. and Koffler, H. 1948. Corn steep liquor in microbiology. Bacteriological Reviews, 12(4): 297.

16. Lindgren, S.E. and Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Reviews, 7(1-2): 149-163.

17. Ljungh, A. and Wadstrom, T. 2006. Lactic acid bacteria as probiotics. Current Issues in Intestinal Microbiology, 7(2): 73-90.

18. Marteu, P. 2001. Prebiotics and probiotics for gastrointestinal health. Clinical Nutrition, 20: 41-45.

19. Mercenier, A., Pavan, S. and Pot, B. 2003. Probiotics as biotherapeutic agents: present knowledge and future prospects. Current Pharmaceutical Design, 9(2): 175-191.

20. Naidu, A.S., Bidlack, W.R. and Clemens, R.A. 1999. Probiotic spectra of lactic acid bacteria (LAB). Critical Reviews in Food Science and Nutrition, 39(1): 13-126.

21. Prodělalová, J., Španová, A. and Rittich, B. 2005. Application of PCR, rep-PCR and RAPD techniques for typing of *Lactococcus lactis* strains. Folia Microbiologica, 50(2): 150-154.

22. Reid, G., Bruce, A.W., McGroarty, J.A., Cheng, K.J. and Costerton, J.W. 1990. Is there a role for lactobacilli in prevention of urogenital and intestinal infections?. Clinical Microbiology Reviews, 3(4): 335-344.

23. Rolfe, R.D. 2000. The role of probiotic cultures in the control of gastrointestinal health. The Journal of Nutrition, 130(2): 396S-402S.

24. Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. 1976. Bacteriocins of gram-positive bacteria. Bacteriological Reviews, 40(3): 722-756.

25. Walter, J., Heng, N.C., Hammes, W.P., Loach, D.M., Tannock, G.W. and Hertel, C. 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Applied Environmental Microbiology, 69(4): 2044-2051.

26. Yasui, H., Kiyoshima, J., Hori, T. and Shida, K. 1999. Protection against influenza virus infection of mice fed *Bifidobacterium breve* YIT4064. Clinical and Diagnostic Laboratory Immunology, 6(2): 186-192.

Citation: Md. Rafsanul Hoque, Kazi Rasel Uddin, Salina Akter and Md. Nazibur Rahman, 2020. Studies on Probiotic Properties of *Lactococcus lactis* Isolated From Indigenous Yogurt of Local Market in Bangladesh. International Journal of Recent Innovations in Academic Research, 4(2): 24-39.