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Isolation, Characterization and Determination of Antimicrobial Properties of Lactic Acid Bacteria from Human Milk

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Abstract

Breast milk has a distinctive combination of proteins, carbohydrates, minerals, lipids and various vitamins that promote the proper growth, development and immunity of the infants. That's why it is considered to be a complete food for new born babies. Moreover, it is also rich in various bioactive compounds which promote the maturation of immune system as well as develop body's defense against infections. Among these bioactive agents, probiotic bacteria were isolated from human milk in this research work using selective MRS media. Two *Lactobacillus spp.* were isolated from each of the two breast milk samples, were observed as potential probiotics, and identified using morphological and biochemical tests. These bacteria were facultative anaerobic, gram positive, catalase negative and non-endospore forming. They showed tolerance against 0.3% bile concentration and 1-10% NaCl. Sugar fermentation patterns of both isolated bacteria also greatly varied. Isolate-1 from both sample 1 and 2 showed antimicrobial activity against *Shigella flexneri, Shigella dysenteriae, Vibrio cholerae and Salmonella typhi.* Isolate-2 from sample 1 and 2 showed antimicrobial activity against *Shigella flexneri, Shigella dysenteriae, Vibrio cholerae, Salmonella typhi, Staphylococcus epidermidis* and *Pseudomonas* spp. The addition of breast milk probiotics to infant formulas could be a new alternative to mimic some of the functional effects of human milk in children who are not breastfed

Keywords: Human milk, *Lactobacillus* spp., Sugar fermentation pattern, Quantification of organic acid, Bile tolerance, Antimicrobial activity.

1. Introduction

Probiotics are live microorganisms which are defined by the World Health Organization/ Food and Agricultural Organization (2001) as: "Live microorganisms whose administration in adequate amount to the body is able to confer a health beneficial effect on the host". The most common types of microbes which are used as probiotics are lactic acid bacteria (LAB) and Bifidobacteria.

A number of genera within Firmicutes phylum like *Lactobacillus, Lactococcus, Lactosphaera, Carnobacterium, Enterococcus, Streptococcus, Tetragenococcus, Oenococcus, Pediococcus, Weissella, Melissococcus, Vagococcus* constitute lactic acid bacteria.(Ercolini *et al.*, 2001; Jay, 2000; Holzapfel *et al.*, 2001). LAB are Gram-positive bacteria (Fooks *et al.*,1999) able to ferment carbohydrates into lactic acid and energy (Jay, 2000). Some LAB differ in their metabolic pathway for example homofermentative bacteria like *Lactococcus* and *Streptococcus* produce two lactate molecules from one glucose molecule while heterofermentative bacteria like *Leuconostoc* is able to convert one molecule of glucose into ethanol, lactate and carbon dioxide (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers *et al.*, 2000).

Furthermore, lactic acid bacteria yield some organic compounds that contribute to the aroma as well as flavor of the fermented products. (Caplice and Fitzgerald,1999).

Human milk is a complex biological fluid that is species-specific and completely fulfills both nutritional and microbiological requirements of the new born. Breast milk boosts up immune system and builds body defense against various infectious diseases which makes it superior to other food supplements for infants. Various bioactive compounds like immunoglobulins, lysozyme, antimicrobial acids, oligosaccharides, glycoproteins for example lactoferrin, polyamines, immune cells and bioactive peptides present in breast milk that are responsible for it's anti-infective effect (Saavedra JM, 2002; Isaacs CE, 2005). These bioactive compounds of human milk play a major role in the regulation of the anti-inflammatory system. Due to immunomodulatory action of human milk, the incidence as well as severity of various infectious diseases like tetanus, poliomyelitis and diphtheria is lesser in breast-fed infants than those fed with other food formulae (Hahn-Zoric M. *et al.*, 1990). The addition of breast milk probiotics to infant formulas could be a new alternative to mimic some of the functional effects of human milk in children who are not breastfed. That is why breast milk was selected as source of probiotic bacteria in this study. In human milk, most

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frequently occurring LAB are *Lactobacillus*, *Lactococcus* and *Enterococcus* (Federico Lara-Villoslada1 *et al.*, 2007).

Therefore the present research work was undertaken to isolate and characterize lactic acid bacteria from human milk and to study the antimicrobial properties of isolated probiotic bacteria.

2. Materials and Methods

2.1. Collection of samples

Human breast milk used in this study was obtained from two healthy mother volunteers in Khulna Medical College Hospital. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory.

2.2. Media preparation

The lactic acid bacteria Lactobacillus spp. were isolated from breast milk samples using selective MRS broth and MRS agar media (De Man, Rogosa and Sharpe, 1960). Additionally, 0.5% salicin and 0.05% cysteine were added to MRS to improve the specificity of this medium for isolation of Lactobacillus acidophilus and Lactobacillus delb. ssp. bulgaricus, respectively (Hartemink *et al.,* 1997; Lankaputhra *et al.,* 1995; Shah, 2000). The pH of the media was adjusted to 6.5 and 5.2, respectively, using a digital electrode pH meter.

2.3. Isolation of bacteria

Lactobacillus isolate-1 from sample 1 and 2 was obtained using MRS-salicin medium at pH 6.5. One milliliter of each sample was 4-fold serially diluted (l0-1 to l0-4) in 0.15% sterile peptone water. For this agar, the spread-plate technique was used. After solidifying, the plates were incubated anaerobically at 37°C for 24 h. For the isolation of Lactobacillus isolate-2 from the both samples, selective MRS-cystine agar medium at pH 5.2 was used. Then one milliliter of each sample was 4-fold serially diluted (l0-1 to l0-4) in 0.15% sterile peptone water. The plates were incubated anaerobically at 45°C for 72 hr. The cultures were subjected to five sub-cultures to obtain the bacteria in pure culture. The cultures were maintained in MRS broth at pH 6.5.

2.4. Identification

The isolated bacteria were identified as Lactobacillus spp. by observing their morphological characteristics and by means of several biochemical tests.

2.5. Sugar fermentation test

MRS broth at pH 6.5 was put into a screw capped test tube and phenol red (0.01 g per L) was added into the tube as pH indicator. The medium was autoclaved at 121°C for 15 min. After autoclaving, 1 ml of different types of sugar solutions (10%) (filtered and sterilized) were inoculated into the different tube. Then 200 µl of an overnight bacterial culture was inoculated into the broth medium and incubated anaerobically at 37°C for 24 h. As a pH indicator, phenol red was included in the medium; acid production changed the medium from its original color to yellow. After adding the proper amount of broth, Durham tubes were inserted into each culture tube in order to observe gas production.

2.6. NaCl tolerance test

For the determination of NaCl tolerance of isolated lactobacilli, 10 test tube containing MRS broth were adjusted with different concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture of Lactobacillus and incubated at 37°C for 24 h. After 24 h of incubation their growth was determined by observing culture medium turbidity.

2.7. Quantification of organic acid and determination of pH value

One percent (v/v) 24 h active culture of lactobacillus was used to inoculate 10% sterilized skim milk obtained from whole milk (Vita Co-operative Bangladesh Ltd). The initial pH (6.62) was determined by a digital electrode pH meter. The inoculated skim milk was incubated at 37°C for 72 h and samples were collected at 24 h, 48 h and 72 h. Samples having coagulated milk were separated by filtration. The pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was performed through titration with 0.1 N NaOH.

2.8. Bile tolerance activity

Bile tolerance of lactobacilli isolated from human milk was determined using the protocol described by Graciela and Maria (2001).

2.9. Antimicrobial activity of metabolites produced by Lactobacillus isolates

Modified agar well diffusion method of Schillinger and Lucke (1989) and Toba *et al.* (1991) was used to detect antimicrobial activities of cell free supernatant (CFS) produced from the Lactobacillus isolates. These assays were performed in duplicate. Twenty milliliters of nutrient agar medium (MHA, Difco) were poured into each plate. The pathogenic strains (listed in table-8) obtained from bacterial stocks preserved in animal cell culture laboratory of Biotechnology and Genetic Engineering discipline, Khulna University, Bangladesh, were adjusted to109 cfu/mL by adding sterile water, and spread on the surface of nutrient agar plate. Four wells of 4 mm diameter were cut into these agar plates using a sterile tip and 15 µL, 20 µL, and 25 µL of the cell free supernatant (CFS) collected from 72 hour old bacterial culture were poured into three wells and MRS broth was placed in one well as negative control. The pH of the cell free supernatant was 6.1 and the MRS broth was adjusted to the same pH. The plates were incubated aerobically overnight at 37°C. The plates were examined for zones of inhibition.

3. Results

3.1. Identification

Bacteria isolated from human breast milk were identified as *Lactobacillus* spp. by observing their colony morphology, physiological as well as biochemical

characteristics. The isolates which grew on MRS agar *media* with 0.5% salicin and 0.05% cysteine had small, circular, white-creamy color, convex and nontransparent colonies. Microscopically they were Gram-positive, rod shaped, non-motile, catalase negative, and lacked endospores.

3.2. Sugar fermentation pattern

Isolated bacteria were identified up to species level on the basis of their growth at different temperatures and sugar fermentation tests as recommended by Harrigan and McCance (1976). Four isolates were selected from two samples and subjected to sugar fermentation test. In the sugar fermentation patterns, mainly acid and no gas production was observed. The acids and gas production results are presented in Table-1.

Table1.Sugar fermentation patterns of the four isolates from human milk samples

Sample no.	Name of the bacteria isolate	Rib	Sor	Manni	Sucr	Fruct	Cello	Sal	Lact	Gas Production
1	Isolate-1	+	-	+	+	+	+	+	+	no
	Isolate-2	+	-	-	-	+	-	-	+	no
2	Isolate-1	+	-	+	+	+	+	+	+	no
	Iolate-2	+	-	-	-	+	-	-	+	no

Legend. Rib=Ribose, Sor=Sorbitol, Manni=Mannitol, Sucr=Sucrose, Fruct=Fructose, Cello=Cellobiose, Sal=Salicin, Lact=Lactose, (+) means good fermentation and acid production, (-) means no fermentation and no acid production.

3.3. Tolerance to NaCl

The identified lactobacilli (isolate -1 and isolate-2 from sample 1 and 2) from human milk were able to tolerate 1-10% NaCl. The results are presented in Table 2.

Table 2. Tolerance of *Lactobacillus* isolate-1 and isolate-2 to NaCl

Concentration of NaCl (%)	**Isolate-1**		**Isolate-2**	
	Sample-1	**Sample-2**	**Sample-1**	**Sample-2**
1	+++	+++	+++	+++
2	+++	+++	++	++
3	+++	+++	++	++
4	+++	+++	++	++
5	++	++	++	++
6	++	++	+	+
7	++	+	+	+
8	++	+	-	-
9	+	+	-	-
10	-	-	-	-

Legend: (+++) maximal growth, (++) good growth, (+)minimal growth, (-) no growth.

3.4. Quantification of organic acid and determination of pH value

The identified lactobacilli from human milk (isolate-1 and isolate-2 from both samples) coagulated the skim milk and produced organic acids in the sterilized skim milks which were detected by titrimetric methods. The results are presented in Table 3.

Table 3. Organic acids (%) and pH in skim milk produced by *Lactobacillus* isolates from sample-1 and sample-2 of human breast milk

Sample no.	Isolates	Incubation time (Hour)	Incubation temp. (C)	Organic acid (%)	Initial pH of skim milk	pH at end of incubation
1	Isolate-1	24	37^o	2.98	6.61	6.20
		48	37^o	5.225		5.21
		72	37^o	9.178		4.13
	Isolate-2	24	37^o	2.112		6.01
		48	37^o	3.423		6.12
		72	37^o	4.714		5.89
2	Isolate-1	24	37^o	2.90	6.61	6.16
		48	37^o	4.210		5.87
		72	37^o	6.217		3.93
	Isolate-2	24	37^o	2.523		6.01
		48	37^o	3.735		5.96
		72	37^o	5.172		5.96

3.5. Bile tolerance test

Isolated lactobacilli were screened for their ability to tolerate bile salts by spectrophotometery. Results comparing the tolerance of the different isolates to bile salts are presented in Tables 4, 5, 6 and 7.

3.6. Antimicrobial test

Lactobacillus isolate-1 from sample 1 and 2 showed antimicrobial activity against *Shigella flexneri, Shigella dysenteriae, Vibrio cholerae* and *Salmonella typhi. Lactobacillus* isolate-2 from both samples showed antimicrobial activity against *Staphylococcus epidermidis, S. flexneri, S. dysenteriae, V. cholerae, S. typhi,* and *Pseudomonas spp.* Results are presented in Tables 8 and 9 and shown in figures 1 and 2.

Table 4.: Bile salt tolerance of *Lactobacillus* isolate-1 from sample-1 in MRS broth

Concentration of bile salt (%)														
0.05			0.1			0.15			0.2			0.3		
Incubation time (hour)														
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24
Spectrophotometer reading														
1.02	1.927	2.196	1.055	1.940	2.156	1.044	1.974	2.214	0.987	1.901	2.214	0.894	1.887	2.135

Table 5. Bile salt tolerance of *Lactobacillus* isolate-1 from sample-2 in MRS broth

Bile salt concentration (%)														
0.05			0.1			0.15			0.2			0.3		
Incubation time (hour)														
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24
Spectrophotometer reading														
0.916	1.886	2.358	0.948	1.961	2.545	1.045	1.935	2.644	1.168	1.948	2.448	1.172	1.960	2.685

Table 6. Bile salt tolerance of *Lactobacillus* isolate-2 from sample-1 in MRS broth

Concentration of bile salt (%)														
0.05			0.1			0.15			0.2			0.3		
Incubation time (hour)														
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24
Spectrophotometer reading														
1.022	1.007	2.101	1.026	1.005	2.131	1.043	1.032	2.147	1.018	1.030	2.116	1.031	1.006	1.851

Table 7. Bile salt tolerance of *Lactobacillus* isolate-2 from sample-2 in MRS broth

Bile salt concentration (%)														
0.05			0.1			0.15			0.2			0.3		
Incubation time (hour)														
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24
Spectrophotometer reading														
1.104	1.950	2.711	1.276	1.974	2.521	1.144	1.948	2.440	1.220	1.933	2.180	1.238	1.916	2.246

Table 8 . List of the pathogenic bacteria used in antimicrobial assay

Bacteria no.	Name of the bacteria
1	*Staphylococcus aureus*
2	*Staphylococcus epidermidis*
3	*E. coli*
4	*Shigella flexneri*
5	*Shigella dysenteriae*
6	*Vibrio cholera*
7	*Enterococcus faecalis*
8	*Salmonella typhi*
9	*Pseudomonas spp.*

Source: Animal cell culture laboratory of Biotechnology and Genetic Engineering discipline, Khulna University, Bangladesh.

Table 9. *In vitro* antibacterial activity of *Lactobacillus* isolates

Bacterial strains	Diameter of zone ofinhibition in mm					
	Isolate-1 from sample 1 and 2			Isolate-2 from sample 1 and 2		
	Vol. 15µl / well	20µl / well	25µl / well	15µl / well	20µl / well	25µl / well
1.*Staphylococcus aureus*	Nil	Nil	Nil	Nil	Nil	Nil
2.*Staphylococcus epidermidis*	Nil	Nil	Nil	9	16	17
3. *E. coli*	Nil	Nil	Nil	Nil	Nil	Nil
4. *Shigella flexneri*	13	15	18	15	17	19
5. *Shigella dysenteriae*	10	16	17	14	17	22
6. *Vibrio cholera*	13	18	9	13	17	22
7. *Enterococcus faecalis*	Nil	Nil	Nil	Nil	Nil	Nil
8. *Salmonella typhi*	13	17	20	7	13	15
9. *Pseudomonas spp.*	Nil	Nil	Nil	15	16	19

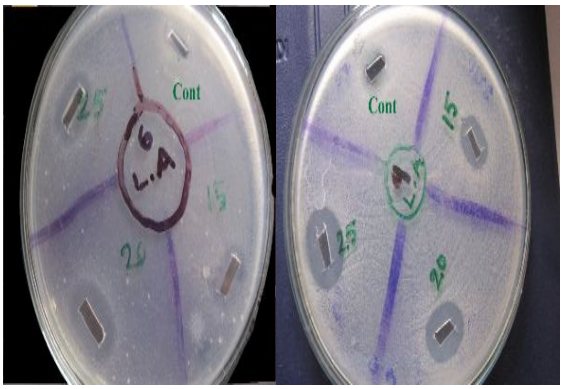

Figure 1. Antimicrobial activity of *Lactobacillus* isolate-1 against *Vibrio cholera* and *Shigella flexneri* (fromleft to right). The wells depicted as Cont in each plate contained blank MRS media as negative control.

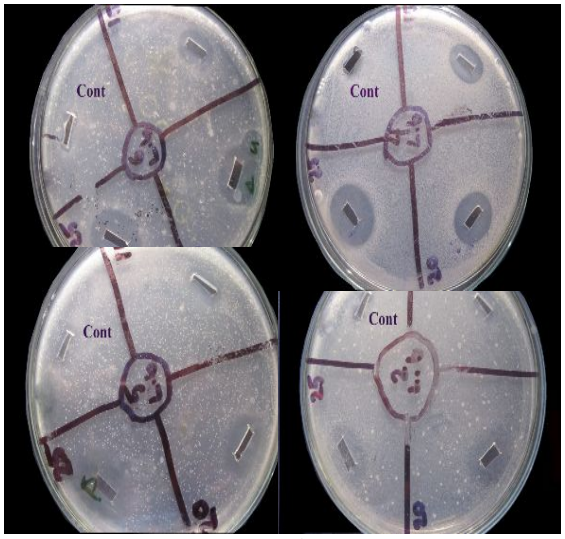

Figure 2. Antimicrobial activity of *Lactobacillus* isolate-2 against *Shigella flexneri*, *Vibrio cholera*, *Shigella dysenteriae* and *S. epidermidis* (clockwise from upper left side). The wells depicted as Cont in each plate contained blank MRS media as negative control.

4. Discussion

On the basis of colonal, morphological and biochemical characteristics (gram positive, catalase negative, endospore absence, non-motile, tolerance to inhibitory substances e.g., 1-10% NaCl, milk coagulation activities, sugar fermentation pattern, bile tolerance activity and antimicrobial activity), the isolates were identified as *Lactobacillus* spp.. The colonies of *Lactobacillus* isolate-1 from sample 1 and 2 are presumed to be *Lactobacillus acidophilus* and appeared rough, dull white, 0.1-0.5 mm in diameter, and demonstrated medium to short rods. The colonies of *Lactobacillus* isolate-2 from both samples are presumed to be *Lactobacillus delbrueckii* ssp. Bulgaricus and appeared cottony, rough, irregular, white,1.0 mm in diameter, and demonstrated long rods in chains. Earlier studies by Vamanu *et al.* (2005), Emanuel *et al.* (2005), Lilia et al. (2002), Oyetayo (2004), and Eduardo et al. (2003) have found similar aforementioned characteristics in isolated lactobacilli.

Isolate-1 from both samples tentatively identified as *Lactobacillus acidophilus* and isolate-2 (tentatively identified as *Lactobacillus delbrueckii* ssp. Bulgaricus) did not produce gas from glucose and other sugars and also showed variation in sugar fermentation patterns. Lactobacillus isolate-1 fermented all the sugars used except sorbitol. On the other hand, *Lactobacillus* isolate-2 from sample 1 and 2 fermented only three sugars (ribose, fructose, lactose) among eight sugars. This observation is consistent with the studies of Shah *et al.* (2000), and Azizpour *et al.* (2009), who found similar fermentation patterns in their isolated lactobacilli.

NaCl is an inhibitory substance which antagonizes the growth of certain types of bacteria. All of the isolates were able to grow at 1-7% NaCl concentration. Isolate-2 did not grow at 8 %, 9% and 10% NaCl concentration, however, isolate-1 grew at these concentrations. Elezete and Carlos (2005) isolated lactobacilli from gastrointestinal tract of swine that were tolerable to 4-8% NaCl. Schillinger and Lucke (1987) were able to grow lactobacilli isolated from meat and meat products in the presence of 7.5% NaCl and these results are similar to the findings of this present study.

Results have shown that organic acid production increased with the incubation time while pH of the media decreased with the increasing acid production. After 72 h incubation at 37°C, highest acidity (9.178%) and lowest pH (4.13) were observed for *Lactobacillus* isolate-1 from sample 1 and highest acidity (4.714%) and lowest pH (5.89) were observed for *Lactobacillus* isolate-1 from sample 2. On the other hand, after 72 h incubation at 37°C, highest acidity (6.217%) and lowest pH (3.93) were observed for *Lactobacillus* isolate-2 from sample 1 and highest acidity (5.172%) and lowest pH (4.89) were observed for *Lactobacillus* isolate-2 from sample 2. These findings are consistent with previous research findings of Haddadin *et al.* (2004), and Rashid *et al.* (2007).

Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h (Prasad, *et al.*, 1998). According to our findings, all of the study isolates are able to grow up in 0.05-0.3% bile salts.

The capacity of substances to inhibit microbial growth is referred to as antimicrobial activity. Isolate-1 showed antimicrobial activity against *S. flexneri*, *S. dysenteriae*, *V. cholerae* and *S. typhi*. Diameter of zones of inhibition ranged from 10 mm to 20 mm. Isolate-2 showed antimicrobial activity against *S. epidermidis*, *S. flexeneri*, *S. dysenteriae*, *V. cholerae*, *S. typhi* and *Pseudomonas* spp. Diameter of zones of inhibition ranged from 7 mm to 22 mm. As the isolated lactic acid bacteria inhibited these pathogenic strains successfully, it may be expected that addition of these human milk probiotics to commercial food products for infants would confer effective protection against infections caused by these pathogens.

5. Conclusion

Lactic acid bacteria were isolated from human milk in pure culture and various properties of isolated bacteria were determined. All of isolates showed tolerance to bile salt, organic acid production and antimicrobial activity against some indicator microorganisms. Phenotypic identification effectively differentiated the isolates especially sugar fermentation patterns. Two different isolate strains were identified and these could be used as potential probiotic strains.

6. Recommendations

Future research work regarding adhesion to mucosal surface, clinical studies for human health, strain stability, bacteriophage resistance, viability in products, antibiotic resistance should be carried out.

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