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ORIGINAL ARTICLE

Assessment of Probiotic Properties of Isolated Lactic Acid Bacteria from Human Milk Sample

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ABSTRACT
Accomplishment of recognizing potential probiotic properties of isolated lactic acid bacteria from human milk was the desired effect of this study. For this perspective collected human milk's sample were nurtured in MRS media with continuous subculture to procure potential probiotic bacteria. From the pure culture, isolated five species of Lactic Acid Bacteria (LAB) namely Lactobacillus acidophillus, Lactobacillus plantarum, Bifidobacterium longum, Enterococcus faecium and Pediococcus acidilactici were characterized using morphological, biochemical and sugar fermentation tests. All isolates displayed coagulation in cow milk and tolerance against 1 - 8% NaCl, 0.1% - 0.4% phenol and 0.05% - 0.3% bile salt concentrations, indicating they are able to survive in adverse condition in intestine. Isolated strains (S1 and S5) showed well antagonistic activity against 4 different pathogens like V cholera, Shigella spp., Salmonella spp. and K pneumonia. As human milk possesses various health promoting probiotic bacteria that develop infant body's defense against infections, so, isolated probiotics can be added to infant-formula taking their health into account.
Keywords; *Probiotics, Lactic acid bacteria, Human milk, Infant-formula*

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INTRODUCTION

Probiotics are the live microorganisms which diminish microbial toxic activity and employ positive affection on the gut micro flora, thereby confer beneficial health effects on host when administered in satisfactory amount to the body [1]. Consumption of probiotics is related to various health benefits on human such as improved immune system, fortification against diarrheal diseases, lowering of cholesterol, nosocomial and respiratory tract infections, , attenuation of overt immune-inflammatory disorders and anticancer effects [2]. Lactic Acid Bacteria (LAB) are one of the common microbes used as probiotics within the phylum Formicates, several bacterial genera such as *Lactobacillus, Enterococcus, Pediococcus* and *Lactococcus* are known as LAB [3]. Most LAB are 'Generally-Regarded As-Safe' (GRAS) except a number of opportunistic pathogens. They are used as dietary and therapeutic adjuvant as well as in food processing industry and as preservatives in milk and milk products. They represent antibacterial activities against some microorganisms [4]. Human milk contains certain LAB such as *Lactobacillus, Enterococcus*, *Bifidobacteria, Streptococcus, Pediococcus* etc. [5]. Besides, some bioreactive compounds present in human milk such as antimicrobial acids, oligosaccharides, glycoproteins, immunoglobulins, lysozyme and other immunomodulatory factors having anti-infective and health promoting effect [6].

Human milk is nothing but a species-specific complex emulsion that modulates the immune response in infants and protects neonates against infectious diseases like respiratory diseases, gastrointestinal infections, diarrhea, eczema, inflammatory bowel disease, diabetes and reduced risk of obesity [7, 8]. Besides it is also effective for colon cancer treatment [9].Though human milk acts as vector of transmission of HIV-1, it gives strong protection against this harmful disease [10]. Breast-fed infants have

fewer incidences of various infectious diseases such as poliomyelitis, diphtheria and tetanus than those fed with food formula [11]. For this reason, it can be used as superior sterile and nutritious food for infants. Moreover, LAB isolated from human milk can be used as food formulas alternative source of mother milk who are not breastfed. In developing countries like Bangladesh, among the children who were not currently breastfed, about 53% deaths were found [12]. Therefore, the purpose of this study is to identify probiotic properties of LAB isolated from human milk so that it can be used as supplement to infant formulas.

MATERIAL AND METHODS

Sample Collection

In this study, human breast milk was collected in sterile 2ml centrifuge tubes aseptically from 12 healthy mother volunteers of Jessore Medical College (JMC) Hospital, Sadar, Jessore-7400, Bangladesh. After that, the milk was kept on ice containing bag immediately and transported to the laboratory of department of Genetic Engineering and Biotechnology, Jessore University of Science and Technology, Jessore-7408, Bangladesh as soon as possible for further studies (within 40 minutes). All procedures followed were in accordance with the ethical standards of responsible the university committee on human experimentation. Informed consent was obtained from all mother volunteers for being included in the study.

Isolation of Lactic Acid Bacteria

For isolation of bacteria MRS-agar spread-plate technique was used, 10-fold serial dilution (10^{-1} to 10^{-10}) of each milk sample (1 ml) in 9 ml sterilized peptone water (0.15%) was dissolved. After solidification of agar plates, the diluted samples were spread and incubated at 37°C for 48 hrs. Streaking was done to obtain bacterial single colony. Colonies containing typical characteristics were selected from plates randomly. Then MRS broth was used to maintaining pH 6.5 to store the culture at refrigeration temperature.

Identification of Lactic Acid Bacteria

For the identification of isolated bacteria, the morphological characteristics and several biochemical tests including gram staining, catalase reaction, oxidase test, motility test as well as 10 sugar fermentation tests were performed [13]. Regarding sugar fermentation, MRS broth and Phenol red (0.018 g/L) were poured into the test tubes and autoclaved at 121°C for 15 min. Then filtered and sterilized various sugar solutions (5%) were taken into various tubes. Thereafter 200 µl of overnight cultured bacteria was inoculated into the broth medium while durham tube inserted into tubes for observing gas production. All test tubes were incubated at 37°C for 24 h. If fermentation occurred, the medium changed its real color red to yellow. Sugar solution free culture was kept as negative control while culture free sugar solution was kept as positive control.

Assessment of Probiotic Properties

Milk coagulation and NaCl tolerance test

To determine the milk coagulation pattern, 1% (v/v) culture of isolated bacteria was inoculated into sterilized milk and incubated for 24 hrs at 37°C temperature. If there were lactic acid forming bacteria, they coagulated milk and that was observed. NaCl tolerance test was performed by adding MRS broth with NaCl of various percentages (1-10%) to test tubes and inoculating 1% (v/v) culture of isolated bacteria into sterilized test tubes. Then the growth of isolated bacteria was observed by their produced turbidity [14].

Phenol tolerance test

For the assessment of phenol tolerance of isolated LAB, MRS broth was added to the test tubes with various concentration of phenol (0.1, 0.2% and 0.4%). After sterilization, 1% (v/v) overnight cultured fresh bacteria were inoculated and incubated at 37°C for 24 hrs. Their growth was observed at different hours like 2, 4 and 24 by Spectrophotometer at optimum density of 620 nm. Culture free phenol solution was prepared as control [14].

Bile salt tolerance test

In case of Bile salt tolerance test, MRS broth (pH 6.5) was added to the test tubes with various concentration of phenol (0.05%, 0.15% and 0.3%) and following sterilization, 1% (v/v) overnight cultured fresh bacteria were inoculated and incubated at 37°C for 24 hrs. Therefore the growth was observed at different hours like 4, 8 and 24 by Spectrophotometer at optimum density of 620 nm [14]. Culture free bile salt solution was used as control.

Antimicrobial test

Agar well diffusion method was used for the detection of antimicrobial activities [15]. The cell free supernatant (CFS) of isolated LAB were prepared by centrifugation (at 1200 rpm for 10 minutes) and filtration. 20 ml of sterilized nutrient agar medium (HiMedia, India) was then poured into every plate. The bacterial strains from stocks were spread on the nutrient agar plate. A sterile tip was used to make 5 mm diameter wells into agar plates where 35 µL of CFS of 72 hours old bacterial culture were inoculated into the wells. Finally, the plates were incubated overnight at 37°C and measure the zones of inhibition.

RESULTS AND DISCUSSION

Identification of Lactic Acid Bacteria

A total 12 LAB strains were isolated that formed round, creamy-white colonies on MRS agar plate. Microscopically 7 isolates were rod shaped whereas 5 were cocci shaped. All isolates were gram-positive, catalase-negative, oxidase-negative and non-motile. Seven isolates (S1, S3, S5, S8, S9, S10 and S11) were appeared as gram-positive, catalase negative, rough, dull white and small rods. Earlier studies have been shown similarities for *Lactobacilli* and *Bifidobacterium* [16, 17]. Five isolates (S2, S4, S6, S7 and S12) were appeared as gram-positive, catalase-negative, white and small coccus. This study showed similarity to the study of Foulquié Moreno *et al.* (2006) and Giraffe (2003) [18, 19].

Sugar fermentation result

Isolated bacteria were identified up to species level on the basis of their sugar fermentation patterns. In the sugar fermentation patterns, mainly acid and no gas production was observed. The acids and gas production results are presented in Table 1. For sugar fermentation patterns, all isolates did not produce gas and also showed variation in fermentation. Three isolates (S1, S5 and S11) were fermented in dextrose, sorbitol, sucrose, fructose, maltose, lactose, rhamnose and showed similarity to *Lactobacillus acidophilus* whereas two isolates (S3 and S8) were fermented in dextrose, sorbitol, sucrose, fructose, lactose, mannitol, rhamnose, raffinose that was similar to *Lactobacillus plantarum* [20]. Two isolates (S9 and S10) were fermented in dextrose, sucrose, fructose, maltose, lactose, raffinose, xylose and showed similarity to *Bifidobacterium longum* [21]. Two isolates (S2 and S12) were fermented in dextrose, sucrose, fructose, maltose, lactose, mannitol while three isolates (S4, S6 and S7) were fermented in dextrose, sucrose, fructose, rhamnose, xylose that showed similarity to *Enterococcus faecium* and *Pediococcus acidilactici* respectively [22].

Table 1: Sugar fermentation pattern of isolates

Isolates	Various sugars and their gas production result										
	Dex	Sor	Sucr	Fruc	Mal	Lact	Man	Rham	Raf	Xyl	Gas Production
S1	+	+	+	+	+	+	-	+	-	-	NO
S2	+	-	-	+	+	+	+	-	-	-	NO
S3	+	+	+	+	-	+	+	+	+	-	NO
S4	+	-	+	+	-	-	-	+	-	+	NO
S5	+	+	+	+	+	+	-	+	-	-	NO
S6	+	-	+	+	-	-	-	+	-	+	NO
S7	+	-	+	+	-	WR	-	+	-	+	NO
S8	+	+	+	+	-	+	+	+	+	-	NO
S9	+	-	+	+	+	+	-	-	+	+	NO
S10	+	-	+	+	+	+	-	-	+	+	NO
S11	+	+	+	WR	+	+	-	+	-	-	NO
S12	+	-	WR	+	+	+	+	-	-	-	NO

Legend: *Dex – Dextrose, Sor – Sorbitol, Sucr – Sucrose, Fruc – Fructose, Mal – Maltose, Lact – Lactose, Man – Mannose, Rham – Rhamnose, Raf – Raffinose, Xyl – Xylose, "+" - good fermentation and acid production while "-" - no fermentation and acid production, WR - Weak Reaction.*

Assessment of Probiotic Properties

Milk coagulation and NaCl tolerance test

All isolates showed coagulation in sterilized cow milk and tolerance of 1-7% NaCl concentration that was observed after 24hrs. The results of NaCl tolerance are represented in Table 2. All isolates showed one of the probiotic properties through coagulation in milk. Haque *et al.* (2010) found that *Lactobacilli* coagulated in milk [14]. On the other side, all of the isolates were able to grow at inhibitory substance like 1-7% NaCl concentration but S2, S4, S8 and S10 isolates grew at 8% while others did not grow. All

isolates did not grow at 9% and 10% NaCl concentration at all. Elezete and Carlos (2005) observed NaCl tolerancy of *Lactobacilli* from gastrointestinal tract of swine were 4-8% [23] and *Bifidobacterium spp.* grew at 6-10% NaCl concentration observed by Collado and Sanz (2007) [24].

Table 2: NaCl tolerance test of isolates

Concentration of NaCl (%)	Isolates											
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
1%	++	++	++	++	++	++	++	++	++	++	++	++
2%	++	++	++	++	++	++	++	++	++	++	++	++
3%	++	++	+	++	+	++	++	++	+	+	++	++
4%	+	+	+	++	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+/-	+	+/-	+	+/-	+/-	+/-	++	+/-	+/-	+	+/-
7%	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
8%	-	+/-	-	+/-	-	-	-	+/-	-	+/-	-	-
9%	-	-	-	-	-	-	-	-	-	-	-	-
10%	-	-	-	-	-	-	-	-	-	-	-	-

Legend: "++" - maximal growth, "+" - good growth, "+/-" - minimal growth, "-" - no growth.

Phenol tolerance test

Isolated LABs showed significant proliferation at 0.1, 0.2 and 0.4% phenol concentration after 2 hrs of growth, however, their growth go down with the increase of phenol concentration. The trending pattern showed resemblance even after 4 and 24 hrs of incubation with enhanced proliferation. Graphical representation of phenol test is depicted in Figure 1. Where, all probiotic isolates were able to tolerate 0.1-0.4% phenol concentration. This result is quietly similar to Sathyabama findings where Probiotic bacteria showed their ability to grow at different phenol concentrations. [25]. This result indicates the isolated bacteria from milk are able to grow in adverse phenolic condition in small intestine.

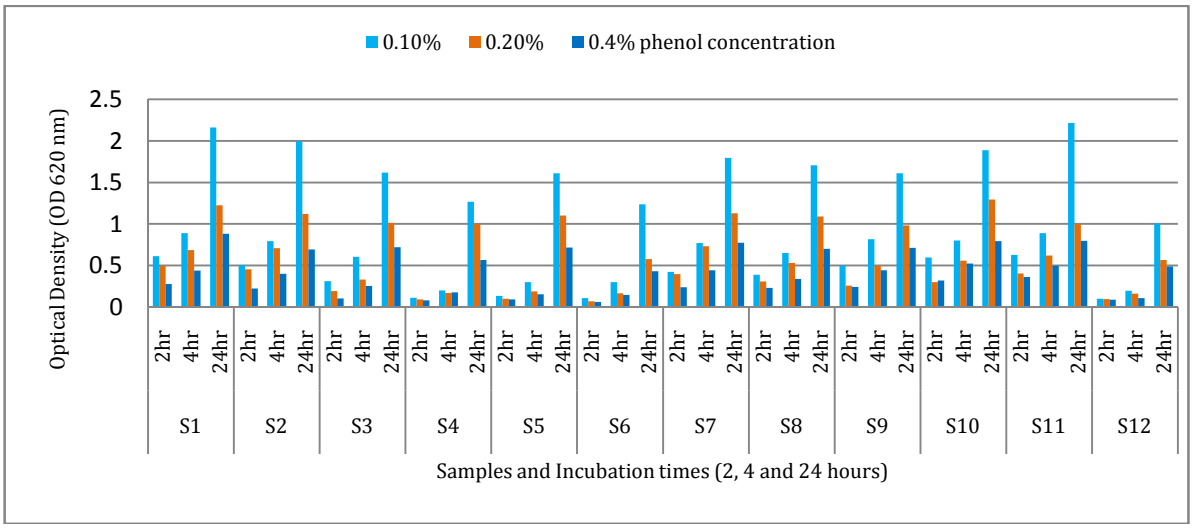

Fig. 1: Phenol tolerance test results–OD620nm values

Bile salt tolerance test

On the other hand isolated strains showed bile salt tolerance at different salt concentration like 0.05%, 0.15% and 0.3% with distinct time interval 4, 8 and 24 hours which are shown in the Figure 2. In this study, all isolates were able to grow up at 0.05-0.3% bile salts. This result is resembled to other researches and assured that isolated strains can survive at bile salt environment in gastrointestinal tract. Begley (2005) and Prasad (1998) showed that 0.3% w/v mean bile salt remains in human gastrointestinal tract and the probiotic strains performed effectively [26, 27].

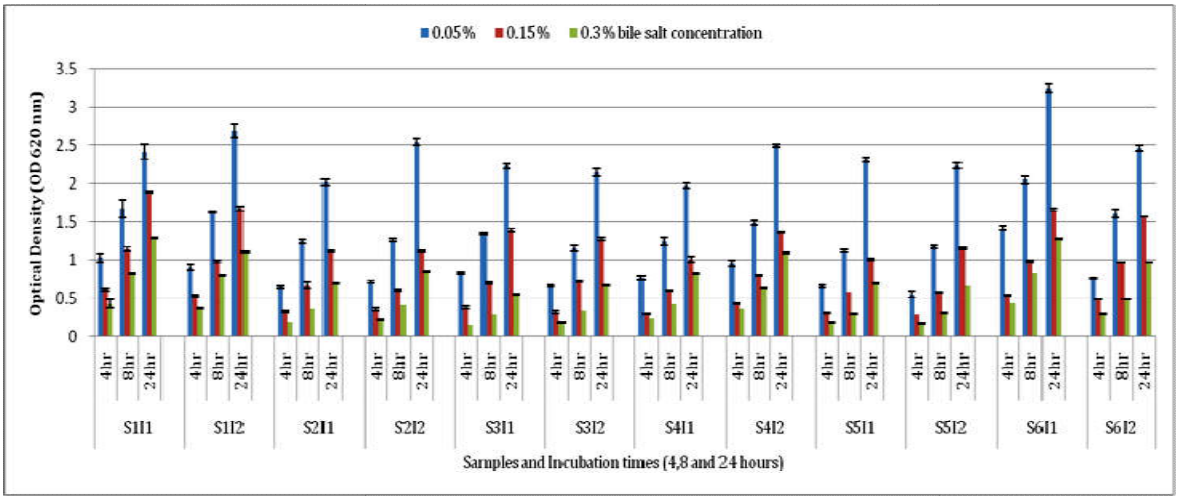

Fig. 2: Bile salt tolerance test results –OD620nm values

Antimicrobial test

Even though the antimicrobial activity for 12 bacterial strains against 8 pathogens was determined, only 8 strains showed antagonistic activity in opposition to 6 pathogenic microbes. The data pertaining to the antimicrobial potential of the isolates is presented in Figure 3. Where, each S1 and S5 isolate exhibited highest antagonistic activity against 3 pathogens like *V cholera*, *Shigella spp.*, *K pneumonia* and *V cholera*, *Salmonella spp., Shigella spp.* respectively. S6 isolate also had antagonistic activity against *Salmonella spp.* and *Shigella spp.* while S2-S4, S8 and S9 isolates showed antimicrobial activity in opposition to 1 different pathogen. Highest zone of inhibition was 19 mm of diameter showed by S5 isolate against *Shigella spp.*, however, there were 4 isolates S7 and S10-S12 did not show any antagonistic activity.

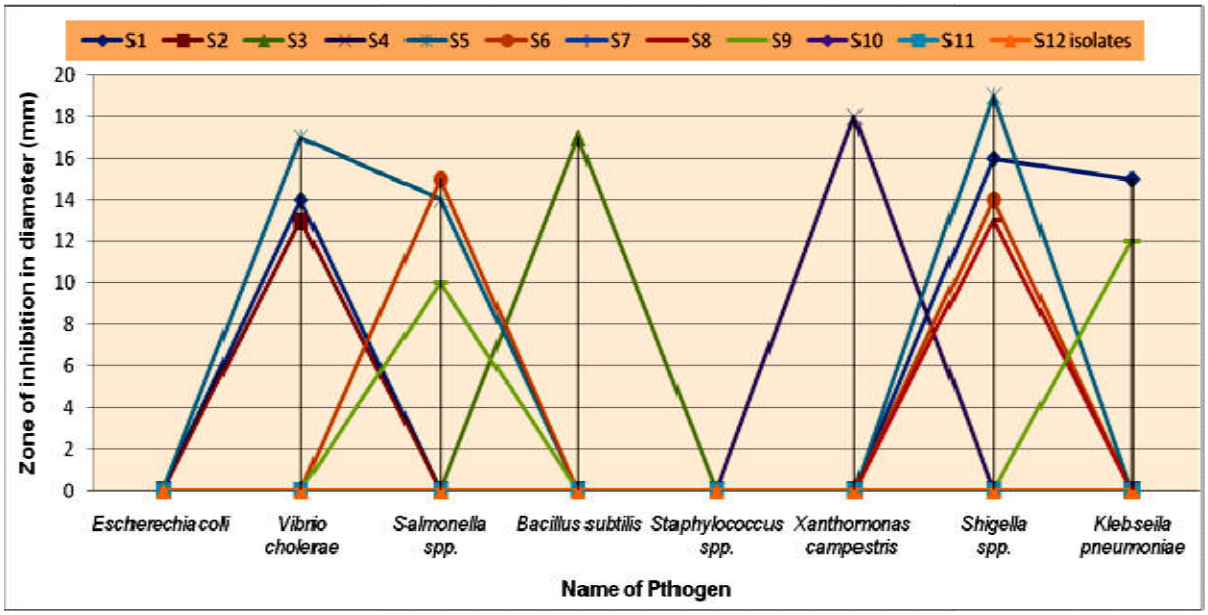

Fig. 3: Antimicrobial test results

CONCLUSION

Among 12 isolates, five species like *Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobacterium longum, Enterococcus faecium* and *Pediococcus acidilactici* successfully identified as LAB. All isolates of the five species showed potential probiotic characteristics including able to coagulate milk and grow well at 1-8% NaCl concentration, 0.1-4% phenol concentration, 0.05-0.3% bile salt concentration and inhibition of 6 pathogenic microbes. They are claimed as probiotic that can be considered as health beneficial. So it is assured that if we use thsese probiotic LAB species as supplement to infant formula fed, it will be very helpful for a child's growth and development through conferring effective protection against pathogenic microbes.

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

ETHICAL APPROVAL
The Authors Declare That This Work Was Not Against Public Interest.

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