Isolation, Identification and Determination of Probiotic potential of Lactic Acid Bacteria from Local Curd

Tasneem Chowdhury* and Saiful Islam

Abstract

Curd is a widely consumed milk product in Bangladesh. Curd samples from sweetmeat stores in Chittagong city of Bangladesh were collected and analyzed for lactic acid bacteria (LAB). Bacterial load of the collected samples was determined by pour plate technique and bacterial count was found in the range of 3.27×10^5 to 1.05×10^6 cfu/mL indicating the samples as excellent nourishing environment. Based on their characteristic growth on MRS agar media six *Lactobacillus* isolates were isolated, similarly six isolates of *Streptococcus* were isolated on YGLA media. Antimicrobial properties of the isolated LABs were evaluated against four human pathogenic bacteria employing modified disc diffusion assay. All twelve isolates exhibited antagonistic activities against at least two or more of the test organisms. In our current study, *Lactobacillus casei* showed antagonism against all test pathogens. Among other *Lactobacillus* isolates *L. xylosus*, *L. homohiochii* and *L. fermentum* inhibited three pathogens, whereas *L. salivarius* and *L. leichmannii* showed inhibition against two pathogens. Among *Streptococcus* isolates, *S. thermophilus*, *S. uberis*, *S. suis*, *S. faecalis and S. equnius* were found to inhibit the growth three test pathogenic bacteria while *S. lactis* was reported to suppress the growth of *E. coli* and *Salmonella paratyphi*. The current study reports some LABs with promising antagonistic properties against test pathogenic bacteria and further investigations on the LABs will validate their appropriateness in using them for improvement of health and services.

Key words: Lactic acid bacteria, *Lactobacillus*, *Streptococcus*, probiotic

INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rod shaped, catalase-negative and fastidious organisms frequently isolated from milk and dairy products. This naturally occurring bacteria are non-pathogenic to human and animals hence 'Generally Recognized as Safe' (GRAS) organisms [8]. LAB have been receiving considerable attention as "probiotics" because of their innate ability to exert antagonistic activity against, non-pathogenic and spoilage organisms [3]. Appreciable numbers of research have been devoted to isolating novel probiotic LAB with emphasis on their health promoting properties and mode of antimicrobial action [7]. Though various methods of delivering probiotics to human intestinal tract are being practiced, dietary supplement through dairy products such as curd, yogurt, ice cream, cheese and other is considered as the best vehicle [6]. Curd is traditional fermented food prepared from cow milk, buffalo milk or standardized milk. Availability of raw milk, easy manufacturing process, low cost and high nutritive value make curd as one of the popular food items in Bangladesh. By acknowledging curd's suitability as dietary agent of providing probiotics to intestine, the current study was designed to analyze some local curd variety for isolation of LAB equipped with probiotic action. Five curd samples were analyzed from which twelve LAB isolates with antimicrobial action had been characterized.

MATERIALS AND METHODS

Collection of samples

Though curd from various commercial producers were available in local markets, five popular and frequently consumed curd verities were collected from different sweetmeat stores of Chittagong. Samples were transported into the laboratory and physicochemical parameters were determined.

Enumeration of Bacteria

Total bacterial load in the samples were determined by serial dilution-pour plate method. Samples were diluted and aliquots were plated on to plate count agar media. After incubation at 37°C for 24 hours, total bacterial load was determined in the form of cfu/mL.

Isolation of LAB

De Man, Rogosa and Sharpe (MRS) agar media was used for selective isolation of *Lactobacillus* [2] whereas Yeast Glucose Lamco Agar (YGLA) media was used for isolation of *Streptococcus* [9]. Bacterial colonies isolated from curd samples were streaked across the two selected media and incubated at 37°C for 48hrs. After incubation colonies developing with characteristic growth characters were isolated and purified. The presumptive isolates were

confirmed as LAB by their morphological properties after gram staining, negative catalase test and ability to produce gas in nutrient broth containing 5% glucose at 37°C within 48hrs.

Characterization and Identification of LAB

With a view to identify the isolates upto species level cultural, morphological, physiological and biochemical characteristics of the isolates were investigated, The observed characters were compared to Bergey's Manual of Determinative Bacteriology, 8th edition [1].

Determination of antibacterial activity of LAB

Antimicrobial activities of the selected isolates were evaluated against *Bacillus cereus*, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC 25922 and *Salmonella paratyphi* AE14613. The four pathogenic test organisms were collected from stock collection of laboratory of Dept. of Microbiology, University of Chittagong. The isolates were grown in nutrient broth at 37°C for 48hrs with constant shaking at 180rpm in a shaking incubator. Following incubation, broth cultures were centrifuged at 7000 rpm for 20 min at 4°C. Cell-free supernatants were collected and soaked in 4mm filter paper discs. Muller-Hinton agar media was uniformly seeded with test organisms and dried paper discs impregnated with culture supernatant were placed on the surface of Muller-Hinton agar media. In control plate, discs soaked with sterile media were placed on the Muller-Hinton agar plate. To ensure proper diffusion of supernatant and to inhibit growth of test organism during the diffusion process, plates were kept in a 4°C refrigerator for 30 min after which the plates were incubated at 37°C for 48hrs. The antibacterial activities of the test agents (supernatants) were determined by measuring the zone of inhibition. The experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Physicochemical characters and total bacterial load of the five collected curd samples were represented in Table1. The pH values of the samples were found within slightly acidic range due to the presence of acid in the sample.

All samples harbored large number of bacteria as milk is an excellent media for growth and proliferation of bacteria.

Twelve presumptive LABs were isolated on MRS and YGLA media. The presumptive isolates validate as LAB because of their gram reaction, negative catalase test and gas production in 5%glucose test.

While observed cultural, morphological, physiological and biochemical characteristics of the isolates were compared with standard descriptions given in Bergey's Manual [1], the isolates were found to be closely related to *Lactobacillus casei* (SM_1), *L. xylosus* (SM_2), *L. homohiochii* (SM_3), *L. salivarius* (SM_4), *L. leichmannii* (SM_5), *L. fermentum* (SM_6), *Streptococcus Lactis* (SY_1), *S. thermophilus* (SY_2), *S. uberis* (SY_3), *S. suis* (SY_4), *S. faecalis* (SY_5) *and S. equnius* (SY_6). Their cultural, morphological and biochemical characteristics are showed in Table 2, Table 3 and Table 4.

The LABs were investigated for their antagonistic activity against two gram positive (*Bacillus cereus* and *Staphylococcus aureus*) and two gram negative pathogenic bacteria (*E. coli* and *Salmonella paratyphi*). The inhibitory effects of cell-free supernatants were evaluated in vitro and shown in Figure 1 and Figure 2.

Antimicrobial activities of the cell-free extract of isolated LABs were assessed against pathogenic *Bacillus cereus, S. aureus, E. coli* and *S. paratyphi* using disc diffusion method. Antagonistic activities of the isolated *Lactobacillus* spp. were interpreted in the form of diameter of inhibitory zone in disc diffusion assay and presented in Figure 1. Among the six isolated *Lactobacillus* spp., *L. casei* was found to inhibit all four test pathogens. However, the other isolates did not show antagonism against all test pathogens. *L. xylosus* inhibited growth of *B. cereus, E. coli* and *S. paratyphi* but failed to exhibit any growth retarding ability against *S. aureus*. Although *L. homohiochii* inhibited *E. coli* and S. *paratyphi* but did not show any antagonism against *B. cereus*. In the case of *L. salivarius*, two of the four organisms, *S. aureus* and *S. paratyphi*, were sufficiently inhibited. *B. cereus* and *E. coli* culture was inhibited by *L. leichmannii* extract but against *S. aureus* and *S. paratyphi*, no antimicrobial activity was reported in our study. Besides, culture extract of *L. fermentum* showed antagonism against three pathogens except *E. coli.* As per Figure 1 culture supernatant of *L. casei* was found to be the most prominent inhibitor of B. cereus. Growth of *S. aureus* and *S. paratyphi* was mostly inhibited by *L. salivarius* while growth of *E. coli* in culture was predominantly suppressed by *L. leichmannii*.

On the other hand, according to Figure 2, *Bacillus cereus* was mostly suppressed by *S. thermophilus* whereas *E. coli* was mostly inhibited by *S. lactis*. However, growth of both *Staphylococcus aureus* and *Salmonella paratyphi* was predominantly suppressed by *S. equnius*. All isolated *Streptococcus* spp. except *S. lactis*, showed antibacterial activities against three pathogens. *S. lactis* extract inhibited only two pathogens, *E. coli* and *Salmonella paratyphi* but showed no evidence of antagonism against remaining pathogens. The maximum inhibition of *S. lactis* was observed against *E. coli*. Though, cell free culture extracts of *S. thermophilus* and *S. faecalis* both were suppressed by *B. cereus* culture predominantly.

Lactic acid bacteria generally show antibacterial activity against many pathogenic and spoilage bacteria. A number of studies reported antimicrobial activities of *Lactobacillus* spp. against entero- and uro- pathogens [5]. Suppression of Gram positive bacteria by *Streptococcus* isolates has also been well documented. Several mechanisms have been attributed to explain antagonistic activities of LABs [4], [10]. Lowering pH of the harboring environment by producing lactic and/or acetic acid, competition for nutrients and adhesion sites with other inhabiting bacteria in surroundings, production of bacteriocins and antioxidants are the most pronounced mechanisms of antibacterial actions. Due to their ability to exhibit antagonism against pathogens and spoilage microorganisms, LABs have been emerging as promising protective agents of food and health. Many authors described protective effects of LAB in boosting of the immune system, inhibition of the growth of pathogens, prevention of diarrhea from various causes, prevention of cancer, reduction of the risk of inflammatory bowel movements, improvement of digestion of proteins and fats, synthesis of vitamins, and detoxification and protection from toxins. The twelve LAB isolates in our study had been found to active against some test pathogens. The results suggest potentiality of the isolates to use them as health protective and promoting agents.

CONCLUSION

In this current study, twelve LABs were isolated from local curd and their antagonistic activities against four human pathogens were observed. As curd is a popular dairy product in Bangladesh being consumed frequently at home and in occasions, consumers are expected to receive beneficiary probiotic actions from such food items. Further characterization of the isolates focusing on their suppressive mechanism as well as checking suitability of using them in industrial scales are needed prior to exploit them for intended use.

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Table 1. The type, color, pH and total bacterial load of the collected samples

No. of sample	Type	Color	pH	Total bacterial count (cfu/mL)
1	Shokti Dahi	Whitish	6.1	1.05×10^6
2	Curd	Cream	6.0	3.37×10^5
3	Curd	Whitish	6.3	2.25×10^5
4	Curd	Whitish	6.0	3.01×10^5
5	Curd	Whitish	6.3	3.9×10^5

Table 2. Colony morphology of the isolates

Isolates	Cultural Characters				Slant Characters
	Color	Elevation	Margin	Form	
SM_1	Whitish	Flat	Entire	Circular	Echinulate
SM_2	Whitish	Convex	Entire	Circular	Filiform
SM_3	Whitish	Convex	Entire	Circular	Echinulate
SM_4	Whitish	Convex	Entire	Circular	Echinulate
SM_5	Whitish	Round	Entire	Circular	Echinulate
SM_6	Whitish	Umbonate	Filamentous	Filamentous	Umbonate
SY_1	Whitish	Convex	Entire	Circular	Beaded
SY_2	Cream	Convex	Entire	Circular	Filiform
SY_3	Whitish	Raised	Entire	Circular	Spreading
SY_4	Cream	Convex	Entire	Circular	Echinulate
SY_5	Whitish	Round	Entire	Circular	Beaded
SY_6	Whitish	Raised	Entire	Circular	Echinulate

Table 3. Microscopic features and staining characteristics of the isolates

Isolates	Vegetative cells		Staining	
	Form	Arrangement	Gram	Spore
SM_1	Short rod	Single and in pair	Positive	Non-spore former
SM_2	Rod	Single, pair & in chain	Positive	Non-spore former
SM_3	Short rod	Single & in pair	Positive	Non-spore former
SM_4	Rod	Single, pair & in chain	Positive	Non-spore former
SM_5	Short rod	Single & in chain	Positive	Non-spore former
SM_6	Short rod	Single, pair & in chain	Positive	Non-spore former
SY_1	Cocci	Single & in pair	Positive	Non-spore former
SY_2	Spherical or ovoid	Single, pair & in short chain	Positive	Non-spore former
SY_3	Spherical or ovoid	Single	Positive	Non-spore former
SY_4	Cocci	Single & in pair	Positive	Non-spore former
SY_5	Cocci	Single	Positive	Non-spore former
SY_6	Spherical or ovoid	Single, pair and short chain	Positive	Non-spore former

Table 4. Biochemical characteristics of the isolates

Isolates	Catalase Test	Motility Test	Starch Hydrolysis	Casein Hydrolysis	H_2S production	Indole test	Methyl red test	Voges-Proskauer test (V.P.)	Citrate utilization	Sugar Fermentation Test									
										Glucose	Fructose	Sucrose	Maltose	Lactose	Galactose	Mannitol	Inulin	Raffinose	Xylose
SM_1	-	-	-	-	+	-	-	-	+	A	A	A	-	K	-	-	-	-	-
SM_2	-	-	-	-	+	-	-	-	+	A	A	A	A	A	K	A	A	-	A
SM_3	-	-	-	+	+	-	-	-	+	A	A	-	A	-	-	A	-	-	-
SM_4	-	-	-	-	+	-	-	-	+	A	A	A	A	A	A	A	A	A	-
SM_5	-	-	-	+	-	-	-	-	+	A	A	A	-	K	-	-	A	A	-
SM_6	-	-	+	+	+	-	-	-	+	A	A	A	A	A	A	A	A	A	A
SY_1	-	-	-	-	+	-	-	-	+	A	A	A	-	A	A	A	A	A	-
SY_2	-	-	+	-	-	-	-	-	+	A	A	A	-	A	A	K	-	K	-
SY_3	-	-	+	-	-	-	-	+	+	A	A	A	A	A	A	A	A	A	A
SY_4	-	-	+	-	+	-	-	-	+	K	K	A	A	A	A	K	K	A	K
SY_5	-	-	-	-	+	-	-	-	+	A	A	A	A	A	A	A	-	-	A
SY_6	-	-	+	-	+	-	-	-	+	A	A	A	A	K	A	A	A	A	-

Note: '+' = positive, '-'= negative, A= Acid, K=Alkali

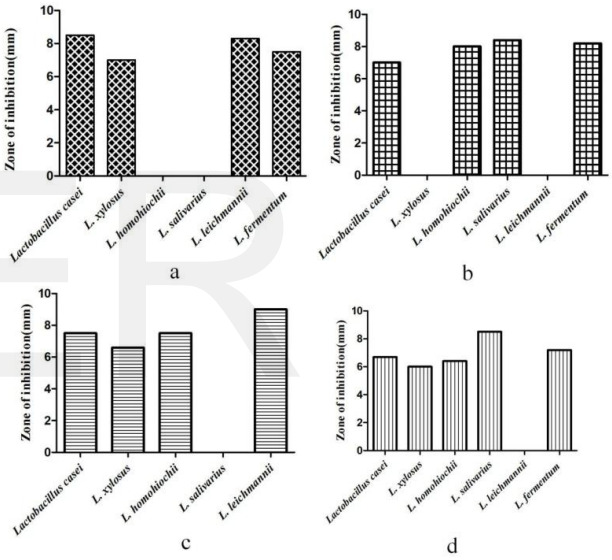

Fig. 1**:** Diameter of zone of inhibition (mm) produced by *Lactobacillus* isolates against four test pathogenic bacteria as assessed by disc diffusion method. a) *Bacillus cereus*, b) *Staphylococcus aureus* , c) *Escherichia coli* and d) *Salmonella paratyphi*

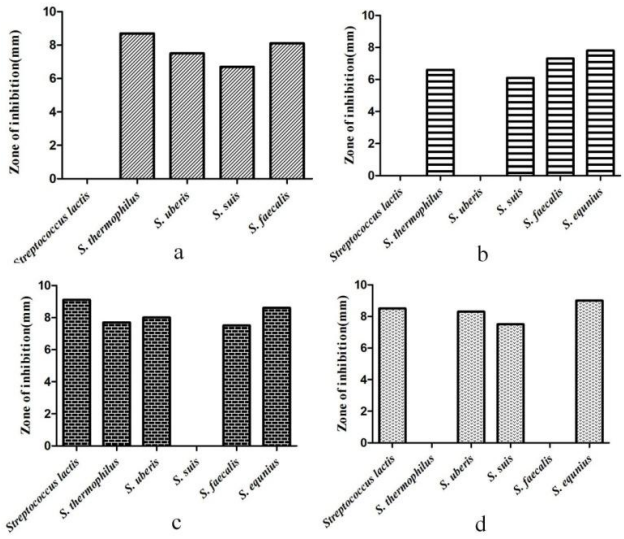

Fig. 2: Diameter of zone of inhibition (mm) produced by *Streptococcus* isolates against four test pathogenic bacteria as assessed by disc diffusion method. a) *Bacillus cereus*, b) *Staphylococcus aureus* , c) *Escherichia coli* and d) *Salmonella paratyphi*