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Isolation and Biochemical Characterization of *Lactobacillus* species from Yogurt and Cheese samples in Dhaka Metropolitan Area

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Abstract

The purpose of this study was to explore *Lactobacillus* species from yogurt and cheese that can be used as potential probiotics. In this study, a total of twenty five samples, fifteen from cheese and ten from yogurt were collected from local markets, Dhaka city during May-July, 2016. Single colonies were isolated by enriching in MRS broth and subsequent streaking on MRS agar plate. Total twenty five isolated bacteria were identified as *Lactobacillus* species by morphological, gram staining and short biochemical tests. All isolated strains were characterized for probiotic properties including acid and salt tolerance, phenol tolerance, sugar fermentation, lactose fermentation and proteolytic activity. Acid tolerance test was performed at pH 2, 3, 4, 5, 6, 7 and 8 in MRS broth. Results showed all isolates survived in highly acidic pH, however most of the strains also survived in alkaline media (pH 8). Salt tolerance test was performed at 2%, 4% and 8% NaCl in MRS broth. All isolates survived in 2% and 4% NaCl concentrations. Phenol tolerance test was performed in MRS broth with 0.1%, 0.2%, 0.3% and 0.4% phenol concentration. All strains survived in 0.1% and 0.2% phenol concentrations. Sugars such as glucose, fructose, sucrose, xylose and lactose were used for fermentation tests. Results of fermentation test showed that most isolates fermented all sugars. All strains digested casein by producing protease enzyme in skim milk agar plate. This study indicated that *Lactobacillus* species from yogurt and cheese samples have potential probiotic properties. Further study is needed to find specific probiotics with specific benefit from yogurt and cheese.

Key words: *Lactobacillus* spp., probiotic, cheese, yogurt.

Introduction

Microbes associated with various beneficial effects for humans and animals are used as probiotics. These microorganisms contribute to balance in gut flora and play important roles in maintaining health (Schrezenmeir and de Vrese, 2001). The probiotic microorganisms mainly consist of the strains of the genera *Lactobacillus* and *Bifidobacterium*, *Streptococcus* and some *Enterococcus* species (Morrow *et al.*, 2012). These bacteria play an important role in the protection of the organism against harmful microorganisms and strengthen the host's immune system (Soccol, 2010; Pundir *et al.*, 2013). Hutt and colleagues (2006) also reported that these bacteria inhibited enteric and urinary pathogenic bacteria. Some of these L*actobacilli* strains have therapeutic properties including anti-inflammatory (Prado *et al.*, 2008) and anti-cancer activities (Chiang *et al*., 2012). These bacteria are also beneficial in gastrointestinal disturbances including diarrhea, dysentery, typhoid (Tambekar and Bhutada, 2010; Cross, 2002). In Bangladesh, yogurt and cheese, locally known as dahi and ponir, respectively are produced and consumed in large amount in all parts of the country (Shahnaz *et al.*, 2004). Many studies have reported that members of the genus *Lactobacillus* are widely found in dairy foods and play an important role in the manufacturing of different dairy products such as yogurt, cheese and other milk products (Keer *et al.*, 1983; Saarela, 2002; Salminen *et al.*, 1996; Tserovska *et al.*, 2000, 2002). However, despite growing interest in probiotics, there is a paucity of scientific research regarding emerging uses of *Lactobacillus* as probiotics on local cheese and yogurts in Bangladesh. Therefore extensive studies are required for finding *Lactobacillus* probiotics for therapeutic benefit from these milk products. In this

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study, an attempt has been taken to find potential probiotic properties of *Lactobacillus* spp. from yogurt and cheese in Bangladesh.

Materials and Methods

Collection of bacterial samples: Total twenty five samples, fifteen from cheese and ten from yogurt were collected from different local markets in Dhaka during May-July, 2016. 1 gm of sample was taken in 9 ml of MRS Broth (Hi-Media, India) and incubated at 37°C for 48 h. One loopful broth culture was streaked on MRS agar plates and incubated 48 hrs. Suspected single colonies were isolated and identified by gram staining and short biochemical tests (MacFaddin, 2000; Bergey *et al.*, 1994). Single colony was stored in MRS agar slant for further study.

Gram staining: Gram staining test was performed for all isolated strains according to the standard procedure. A smear of single colony was prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat fixed smear was flooded with crystal violet solution and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95% ethyl alcohol and rinsed with water. Finally safranin was used as counter stains for 60-80 sec and washed with water, and examined under oil immersion (100X). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively.

Catalase test: A drop of 3% hydrogen peroxide was added to a fresh culture on a sterile glass slide and mixed well. Producing bubble or froth, indicated catalase-positive and no bubble or froth indicated catalase negative. *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as positive and negative control, respectively.

Kliger's Iron Agar (KIA) test: All isolates were tested for KIA test to know the mode of glucose and lactose utilization. Fresh culture was inoculated by stabbing the butt and streaking the slant. After incubation at 37°C for 24 h, results were recorded for color changes of the butt or slant, H_2S or other gas production. The results were observed as alkaline slant and acid butt for fermentation of glucose only, acid slant and alkaline butt for fermentation of lactose only, acid in both slant and butt for fermentation of both lactose and glucose whereas alkaline in both slant and butt for fermentation of neither lactose nor glucose. Production of hydrogen sulphide made blacking of the medium and the gas production give rise to bubble formation in the tube. *S. aureus* ATCC 25923 was used as positive control.

pH tolerance test: MRS broth at pH 2, 3, 4, 5, 6, 7 and 8 were prepared by adjusted with 10N HCl and 1N NaOH. Fresh bacterial cultures were inoculated into respective MRS broth in test tubes and incubated at 37°C for 48 h. Only media was used as negative control. Results were obtained by observing turbidity of the culture media after 24 h and 48 h and no growth was observed in negative control.

NaCl tolerance test: NaCl tolerance of isolated *Lactobacillus* was determined by using MRS broth with 2%, 4% and 8% of NaCl concentration. Fresh culture was inoculated and incubated at 37°C for 48 h. Only media was used as negative control. Results were determined by observing the turbidity after 24 h and 48 h and no growth was observed in negative control.

Phenol tolerance test: MRS broth containing 0.1%, 0.2%, 0.3% and 0.4% of phenol concentration were prepared for the determination of phenol tolerance. Fresh culture was inoculated and incubated at 37°C for 48 h. Only media was used as negative control. Results were determined by observing turbidity after 24 h and 48 h and no growth was found in negative control.

Determination of sugar fermentation: Sugar fermentation test was performed using 1% (w/v) sugar in MRS broth. Glucose, fructose, sucrose, xylose and lactose were used in this test. Phenol red solution was used as indicator. 10 ml media was dispensed and Durham's tube was inserted invertably in each of test tubes. Fresh culture was inoculated and incubated at 37°C for 24 h. Only media was used as negative control. Results were observed by color changing and gas formation.

Casein digestion test: The protease activity was performed using MRS agar plate containing 1% skim milk solution. Bacterial cultures were inoculated and incubated for 48h at 37°C. Clear zones around the cultures indicated protease activity (Smibert and Krieg,

1994). *Pseudomonas* spp. and *Klebsiella* spp. were used as positive and negative control, respectively.

Results and Discussion

Lactobacilli species from fermented foods are an important source of probiotics. Yogurt is one of the best known foods that contain probiotics (Oskar *et al*., 2004). Therefore, focus of the present study was to find *Lactobacillus* strains from local fermented foods yogurt and cheese in Dhaka. In this experiment, total 25 *Lactobacillus* spp. were identified on the basis of characteristic morphology, catalase negative and gram positive rod shape (Table 1 & 2 and Figure 1 & 2). Previously, *Lactobacillus* spp. were identified and confirmed by gram positive and catalase negative results (Rao *et al.*, 2015; Salvetti *et al.*, 2012; Vyas *et al.*, 2014). However, another report showed that *Lactobacilli* are genetically and physiologically diverse group of rod-shaped, gram-positive, catalase negative bacteria (Hoque *et al*., 2010). Although many studies have reported *Lactobacillus* strains found in human gut including oral cavity. In this study, survivability study in different pH was performed because pH is an important factor for the growth of bacteria. All strains were tested in both acidic and alkaline conditions at different ranges of pH 2 to 8. From this experiment, isolated *Lactobacillus* showed maximum growth at pH 2. Therefore, these bacteria exhibited survival in both highly acidic and moderate alkaline conditions and less growth in relatively neutral pH 6 and 7 (Table 3). In previous study, Pundir R.K. *et al.* (2013) reported that *Lactobacillus* isolated from fresh vegetable, fruits, curds survived in pH 3.5 to pH 7.0. Another study by Hoque M.Z. *et al*. (2010) found *Lactobacillus* growth in pH 2.5 to pH 8.5. The reason for choosing this pH range was to determine whether the isolated strains can grow in both acidic and alkaline conditions. pH

Table 1. Colony morphology of isolated bacteria.

Isolate no.	Morphology of colony	Color of colony
KL1	Small, circular, regular	Creamy
KL2	Small, circular, regular	Creamy
KL3	Large, circular, irregular	Creamy
KL4	Small, circular, irregular	Creamy
KL5	Small, circular, irregular	Creamy
KL6	Small, circular, regular	Creamy
KL7	Small, circular, regular	Creamy
KL8	Small, circular, regular	Creamy
KL9	Small, circular, regular	Creamy
KL10	Small, circular, regular	Creamy
KL11	Small, circular, regular	Creamy
KL12	Small, circular, regular	Creamy
KL13	Large, circular, regular	Creamy
KL14	Small, circular, regular	Creamy
KL15	Small, circular, regular	Creamy
KL16	Large, circular, regular	Creamy
KL17	Small, circular, regular	Creamy
KL18	Small, circular, regular	Creamy
KL19	Small, circular, regular	Creamy
KL20	Small, circular, regular	Creamy
KL21	Small, circular, regular	Creamy
KL22	Small, circular, regular	Creamy
KL23	Large, circular, regular	Creamy
KL24	Small, circular, irregular	Creamy
KL25	Small, circular, regular	Creamy

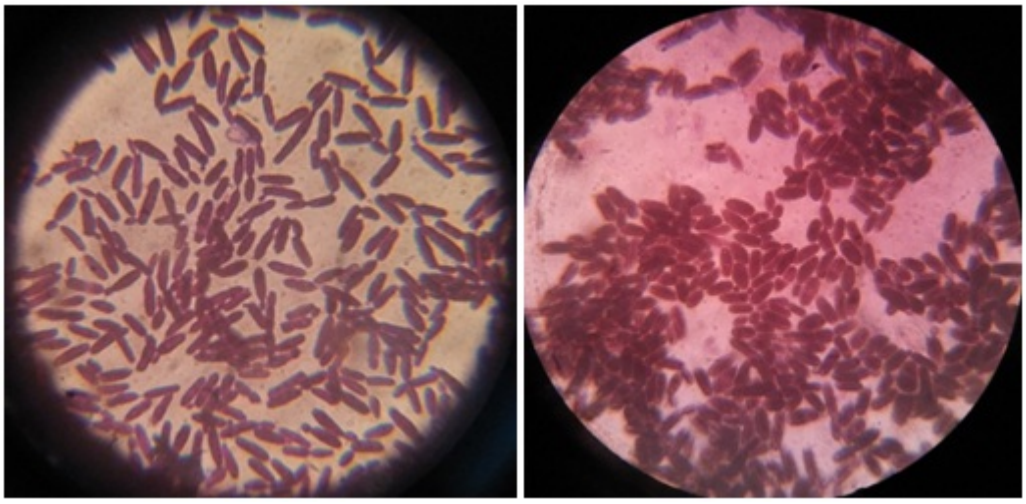
Table 2. Results of biochemical tests of isolated bacteria.

Isolate no.	Gram staining	Catalase	KIA
KL1	Bacilli	-	Alkaline/acid
KL2	Bacilli	-	Alkaline/alkaline
KL3	Bacilli	-	Alkaline/alkaline
KL4	Bacilli	-	Alkaline/alkaline
KL5	Bacilli	-	Acid/acid
KL6	Coccobacilli	-	Alkaline/alkaline
KL7	Coccobacilli	-	Alkaline/alkaline
KL8	Coccobacilli	-	Acid/acid
KL9	Coccobacilli	-	Acid/acid
KL10	Coccobacilli	-	Acid/acid
KL11	Bacilli	-	Alkaline/alkaline
KL12	Bacilli	-	Alkaline/alkaline
KL13	Bacilli	-	Alkaline/alkaline
KL14	Bacilli	-	Acid/acid
KL15	Bacilli	-	Alkaline/alkaline
KL16	Bacilli	-	Alkaline/alkaline
KL17	Bacilli	-	Acid/alkaline
KL18	Bacilli	-	Acid/acid
KL19	Bacilli	-	Acid/acid
KL20	Bacilli	-	Alkaline/alkaline
KL21	Coccobacilli	-	Acid/acid
KL22	Coccobacilli	-	Acid/acid
KL23	Coccobacilli	-	Alkaline /alkaline
KL24	Coccobacilli	-	Alkaline /alkaline
KL25	Coccobacilli	-	Acid/acid

+ = positive; - = negative; KIA: Slant/Butt

tolerance is essential criteria to grow and perform their beneficial effect in the gastrointestinal tract but range of tolerance is not specified. Another test was performed to evaluate NaCl tolerance of L*actobacillus* spp. in this study. NaCl is an inhibitory substance that inhibited the growth of some bacteria (Hoque *et al.*, 2010). NaCl tolerance test was performed at 2%, 4% and 8% NaCl concentrations. All the isolates were able to grow at 2% and 4% NaCl concentration but did not grow at 8% NaCl (Table 3). This is in agreement with Forhad *et al.* (2015) that *Lactobacillus* species were not able to grow in high NaCl concentrations. Phenol is also an inhibitory compound produced in deamination reaction of amino acids in intestine (Suskovic *et al.*, 1997). Probiotic bacterial strains survive low concentrations of phenol. In the present study, phenol tolerance test was performed in 0.1%, 0.2%, 0.3% and 0.4% of phenol concentration. All the isolates survived at 0.1% and 0.2% concentration while 50% isolates survived in 0.3%-0.4% phenol concentrations. A similar report was published by Hoque (2010), where most *Lactobacillus* strains survived up to 0.3% phenol.

Figure 1. *Lactobacillus* species in MRS plate.



(i) Bacilli (ii) Coccobacilli

Figure 2. Gram positive *Lactobacilli* species in gram staining test.

Table 3. Results of NaCl and pH tolerance tests of isolated *Lacobacillus* species.

Isolate	pH 2		pH 3		pH 4		pH 5		pH 6		pH 7		pH 8		NaCl concentration		
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	2%	4%	8%
KL1	+++	+++	++	++	+	+	+	++	-	+	+	+	++	++	+++	+	-
KL2	+++	+++	++	++	+	++	+	++	-	+	-	+	++	++	+++	+++	+/-
KL3	+++	+++	++	++	+	+	+	++	-	+	-	+	++	++	+	+++	-
KL4	+++	+++	++	++	+	+	+	++	-	+	-	-	++	++	+++	+++	+/-
KL5	+++	+++	++	++	+	++	+	++	-	-	-	+/-	++	++	+++	++	-
KL6	+++	+++	++	++	+	++	+	++	-	+	-	+	++	++	++	+	-
KL7	+++	+++	++	++	+	+	++	++	-	+	-	+	++	++	+++	+	+/-
KL8	+++	+++	++	++	+	++	++	++	-	+	-	+	++	++	+++	++	-
KL9	+++	+++	++	++	+	+	++	++	-	+	-	+	++	++	+++	++	+/-
KL10	+++	+++	++	++	+	++	++	++	-	+	-	+	++	++	+	++	-
KL11	+/-	+++	+	++	+	++	+	+	-	+	+	+	++	++	++	+++	+
KL12	+/-	+++	++	++	+	-	+	+	-	+	+/-	+	++	++	++	+++	-
KL13	+++	+++	+	++	+	++	+	++	-	+	+/-	-	++	++	+++	++	-
KL14	+++	+++	++	++	+	+	++	+	-	+	+/-	-	++	++	+++	+++	+
KL15	+/-	+++	+	++	+	+/-	+	++	-	+	+/-	+	++	++	+++	++	-
KL16	+++	+++	++	++	+/-	+	+	++	-	+	+/-	-	++	++	+++	+++	+/-
KL17	+++	+++	++	++	+/-	+	++	++	-	+	+/-	-	++	++	+++	+++	-
KL18	+++	+++	++	++	+	+	+	+	-	+	+/-	-	++	++	+++	+/-	-
KL19	+++	+++	++	++	+	+	+	++	-	+	+/-	-	++	++	+++	++	-
KL20	+++	+++	++	++	+	+	+	+	-	+	+	+	++	++	+++	++	-
KL21	+++	+++	++	++	+	+/-	+	+	-	+	+	+	++	++	++	+	+/-
KL22	+++	+++	++	++	+	+	+	+	-	+	+	+	-	-	+	+++	-
KL23	+++	+++	++	++	+	+	++	++	-	+	+	+	++	++	++	+	+/-
KL24	+++	+++	++	++	+	+/-	+	+	-	+	+	+	++	++	+++	+/-	+/-
KL25	+++	+++	+	++	+	+/-	++	+	-	+	+	+	++	++	+	++	-

- = no growth (negative); + = slight growth (positive); ++ = moderate growth (positive);
+++ = dense growth (positive); +/- = ambiguous/undecided

Table 4. Results of sugar fermentation and casein digestion tests of *Lacobacillus* species.

Isolate no.	Glucose	Fructose	Sucrose	Xylose	Lactose	Casein digestion
KL1	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^+	+
KL2	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL3	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL4	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL5	A^+,G^+	A^-,G^-	A^-,G^-	A^+,G^+	A^+,G^+	+
KL6	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^+	+
KL7	A^+,G^+	A^-,G^-	A^-,G^-	A^+,G^+	A^+,G^+	+
KL8	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL9	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL10	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL11	A^+,G^+	A^-,G^-	A^-,G^-	A^+,G^+	A^+,G^+	+
KL12	A^+,G^+	A^-,G^-	A^-,G^-	A^-,G^-	A^+,G^+	+
KL13	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL14	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL15	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL16	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^-	+
KL17	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL18	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^+	+
KL19	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^+	+
KL20	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^+	+
KL21	A^+,G^+	A^+,G^+	A^-,G^-	A^+,G^-	A^+,G^+	+
KL22	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^+	+
KL23	A^+,G^+	A^+,G^+	A^-,G^-	A^+,G^+	A^+,G^-	+
KL24	A^+,G^+	A^-,G^-	A^+,G^+	A^-,G^-	A^+,G^+	+
KL25	A^+,G^+	A^+,G^+	A^+,G^+	A^+,G^-	A^+,G^+	+

A^+ = Positive Acid; A^- = Negative Acid; G^+ = Positive Gas; G^- = Negative Gas; + = positive

Lactobacillus species is a well known food fermenter worldwide. *Lactobacillus* bacteria fermented different monosaccharide and disaccharide (Pyar and Peh, 2014). In this study, the results of sugar fermentation were shown in table 4. All the isolates fermented glucose and lactose with evolution of gas. 92%, 80% and 76% of isolates fermented xylose, fructose and sucrose, respectively. The probiotic bacteria were capable of fermenting different sugars and end product is lactic acid (Klaenhammer and de Vos, 2011). This is especially useful for lactose intolerant people who cannot metabolize lactose due to lack of β-galactosidase enzyme. Therefore, fermented foods under study can help lactose intolerant persons to consume milk or lactose containing products. Lactose and dextrose utilization by *Lactobacillus* was confirmed by lactose and glucose utilization test. *Lactobacillus* digests casein in order to grow in milk and subsequently utilize the degradation products (Hayes *et al.*, 2007). In the present study, all the isolated strains were found to digest casein indicating *Lactobacillus* produced protease enzyme. Atanasova *et al.* (2014) reported in their study that, *Lactobacilli* species utilized casein by proteolytic activity.

Conclusion

In this study, *Lactobacillus* species were isolated and identified from local cheese and yogurt and they can survive in extreme acidic and moderate alkaline conditions as well as in high concentrations of salt and low concentrations of phenol. These findings revealed that the isolated *Lactobacillus* strains are suitable to survive in the environment of human gastrointestinal tract. However, the strains also have probiotic potential for sugar fermentation, lactose intolerance and casein digestion. Further studies are needed in order to find specific probiotics with specific benefit from yogurt and cheese.

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