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Development of probiotic milk drinks using probiotic strain isolated from local yogurt

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ABSTRACT

Two *Lactobacillus* spp. were isolated from four yogurt samples, which were identified on the basis of their colony morphologies grown on MRS (Man, Rogosa and Sharpe) media and on the basis of biochemical tests conducted in the laboratory. Based on biochemical characteristics and further screen, these two isolates were selected for probiotic screening and further study. Gram staining, Catalase test, Oxidase test, Sugar fermentation, Bile salt test, NaCl tolerance test and pH tolerance test were done in this regard. Based on the OD (Optical Density), it was evident that all isolates can grow from bile salt concentration 0.05% to 0.25% and pH 2.5 to pH 5.5, can be used as probiotic. For NaCl tolerance test, two isolates showed slight decrease of growth during 1% to 4% NaCl supplementation. After isolation of probiotic strain from yogurt, these strains were used for the development of probiotic milk drinks. These drinks were developed where one was controlled and other two were mixed with different concentration of fruit (mango juice). The analysis of probiotic milk drinks showed the highest fat content was 4.50%, protein content 3.99%, ash content 1.90%, acidity 0.78%. Storage studies were carried out for fifteen days at refrigeration temperature (5±10 °C) and samples were taken at an interval of three days. After 15 days acidity ranged between 0.91 to 0.95% and the counts of Isolate 1 and Isolate 2 ranged between 8.75~9.55 log cfu/ml those were >5 log cfu/ml which makes probiotic milk drinks as health product. The sensory scores of the 10% mango juice mixed with probiotic milk drinks was significantly higher than that of the 5% (p<0.05) and the liking score ranged in the medium-like.

Keywords: Lactic acid bacteria, probiotic, milk drinks, yogurt, mango juice

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1 Introduction

Nowadays, consumers are increasingly demanding foods with special properties, such as pleasant flavour, low-calorie value or low fat content, and beneficial health effects. Within this context, Food companies are increasingly manufacturing foods with incorporated probiotic bacteria, which fall under the new

category of foods called Functional Foods. Functional dairy products meet various nutritional requirements, along with health benefits that are strengthened by the addition of probiotics.

The most important and frequently used functional food compounds are probiotics and prebiotics, or they are collectively known as 'synbiotics'. According to [FAO/WHO \(2002\)](#) report, probiotics are

'live microorganisms which when administered in adequate amounts conferring a health benefit on the host'. Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Helland et al., 2004). Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal micro flora. *Lactobacillus* is one of the most important genera of LAB (Coeuret et al., 2003). They are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks (Coeuret et al., 2003).

Lactobacilli comprise a large and diverse group of gram positive, non-spore forming, catalase negative rod bacteria, able to produce lactic acid as the main end product of the fermentation of carbohydrates (Pelinescu et al., 2009; Malek et al., 2012). They are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary applications (Fooks et al., 1999; Dunne et al., 2001). Fruits and vegetables have been suggested as ideal media for probiotic growth because they inherently contain essential nutrients, they are good-looking and have good taste (Luckow and Delahunty, 2004; Sheehan et al., 2007). There is a genuine interest in the development of fruit and milk based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods (Tuorila and Cardello, 2002; Yoon et al., 2004; Sheehan et al., 2007). In addition to providing consumers options for improving their health and well-being, functional foods such as probiotics in dairy products are an attractive market sector, providing new economic opportunities. Probiotics should not adversely affect the taste or aroma of the product or acidification during the shelf life of the product. Flavor is a crucial characteristic of foods as the sensory properties play an important role in product acceptance by consumers.

The objectives of this study were Isolation of Lactic Acid Bacteria (LAB) from yogurt samples available in K.R. market, screening the isolates for various probiotic characteristics for determination of their probiotic potential, development of milk drinks using isolated probiotic strains and studies the shelf life of developed probiotic milk drinks.

2 Materials and Methods

2.1 Isolation of probiotic strain

2.1.1 Collection of sample

Lactic acid bacteria for probiotic characteristics were isolated from different yogurts available in K.R. market. During sample collection sterile paper bag, marker pen, note book etc were taken for the purpose of sampling. Soon after collection, the samples were transported in sample box with ice to the laboratory and maintained at 5 ± 1 °C till the sample preparation started.

2.1.2 Isolation of lactic acid bacteria

Yogurt samples were collected from the K.R. market and then immediately stored at 5 ± 1 °C until sample preparation for isolation. The lactic acid bacteria were isolated from yogurt samples by using selective MRS DeMan et al. (1960) agar media. Plating out technique was used for isolation of *Lactobacillus* spp. The selected colonies were purified using streak plate technique. Finally, the single colony of bacteria was isolated by observing their colonial morphology and some physiological tests (Gram staining, catalase reaction and oxidase reaction).

2.1.3 Carbohydrate fermentation test

The fermentation of carbohydrates including sucrose, maltose, fructose, lactose, dextrose and also sterile water was used as positive and negative controls.

2.1.4 LAB strain resistance to low pH (2.5)

Resistance of isolates for Gastric Juice (*in vitro*) was conducted according to the method of Pennacchia Pennacchia et al. (2004). The survival of isolates was compared in Phosphate-buffered saline (PBS) at pH 5.5 and 2.5.

2.1.5 Bile tolerance

Isolates with most resistance to acid were selected for evaluation of bile tolerance. Bile tolerance was measured as described by Gilliland et al. (1984). Briefly, growth was measured in MRS broth containing 0.05 to 0.25% bile salt in 7 h by spectrophotometer (OD600 nm) and bile salt-free MRS was used as control.

2.1.6 NaCl tolerance

Isolates with most resistance to acid and bile salt were selected for evaluation of NaCl tolerance. Briefly, growth was measured in MRS broth containing 1% to 8% NaCl in 7 h by spectrophotometer (OD600 nm) and NaCl free MRS was used as control.

2.2 Development of probiotic milk drinks

2.2.1 Probiotic culture and cow milk

Isolated culture (Isolate1 and Isolate2) was used as probiotic culture. Whole milk was collected from the dairy farm of Bangladesh Agricultural University, Mymensingh.

2.2.2 Preparation of fruit juice

The fruit (mango) was purchased from Mymensingh town and brought to the laboratory for collecting juice. Firstly, Mango (*Mangifera indica*) fruit was washed by distilled water and the skin was separated with the help of knife using clean hand. Secondly, the fruit pulp of mango was blended. After blending the juice was filtered by clean cloth (hot water washed). They were kept in plastic cups and stored at refrigeration temperature (5 ± 1 °C) until preparation of Probiotic milk drinks.

2.2.3 Preparation of probiotic milk drinks

Milk was heated until its weight reduce to about 20-25%. Sugar was added to the milk after boiling. During heating milk was stirred thoroughly with the help of a stirrer. After desired heating milk pan was taken out from the heater and allowed to cool. When the temperature was about 40 °C, after that milk was divided into three equal portions and three types of Probiotic milk drinks was prepared from each portion by using the different proportions of fruit (mango juice). The fruit (mango juice) was incorporated into whole milk at 5% and 10% level in different cups except control. Milk was inoculated with 2% culture, which was collected, isolated from yogurt. The glass bottles were pre-washed with boiled water before use. The samples were incubated at 37 °C for 8 hrs and then samples were stirred. The properties of milk drinks were determined along 15 days stored period at about 5 ± 1 °C.

2.2.4 Determination of physical, chemical and microbiological properties of the probiotic milk drinks

The viscosity was determined by Brookfield viscometer. Moisture, total solids (TS) and ash content of the different type of samples were determined according to Ranganna (2011). Fat percent was determined by Babcock method using the procedure described by Aggarwala and Sharma (1961). Acidity was determined by titration with N/10 sodium hydroxide solution using the procedure by AOAC (2005). Protein was determined by Kjeldahl described by Ranganna (2011) and AOAC (2005) procedure and pH was measured with the help of a pH meter (HANNA instru-

ments, HI 8424, microcomputer pH meter). For total viable count, standard plate count was done according to the method described in 'Standard Methods for the Examination of dairy products'. The microbial counts were expressed in log cfu/ml.

2.3 Sensory evaluation of the probiotic milk drinks

The consumer's acceptability of developed product taste was evaluated by a testing panel consist of 10 panelists. The hedonic rating test was used to determine the acceptability. The panelists rated their acceptability of the product on hedonic scale.

3 Results and Discussion

3.1 Isolation and screening of lactic acid bacteria for probiotic potential

3.1.1 Morphological characteristics

Based on morphological characteristics, two isolates were detected as lactic acid bacteria. The isolates were grown in MRS medium at pH 6.5. All the isolates produced cream colored, circular, convex, shiny, and moist with smooth edge. All the isolates showed morphologically similar to *Lactobacillus* spp.

3.1.2 Microscopic observation

After gram staining all the isolated microorganisms were identified as rod shaped, a convex, rough, smooth, shiny, irregular, circular, gram positive, facultative anaerobic, non spore forming bacterium which indicates them to be member of *Lactobacillus* spp. (Fooks et al., 1999; Tharmaraj and Shah, 2003; Kandler and Weiss, 1986).

3.1.3 Catalase test

No gas bubble was formed during catalase reaction that means it was catalase negative and it was a confirmation to be *Lactobacillus* spp.

3.1.4 Oxidase Test

During oxidase reaction cytochrome oxidase-negative bacteria did not cause any change in color therefore isolates were oxidase negative which indicates isolates were *Lactobacillus* spp.

3.1.5 Carbohydrate fermentation test

Carbohydrate fermentation test showed the following result and that was the clear indication for both the isolate to be *Lactobacillus* spp.

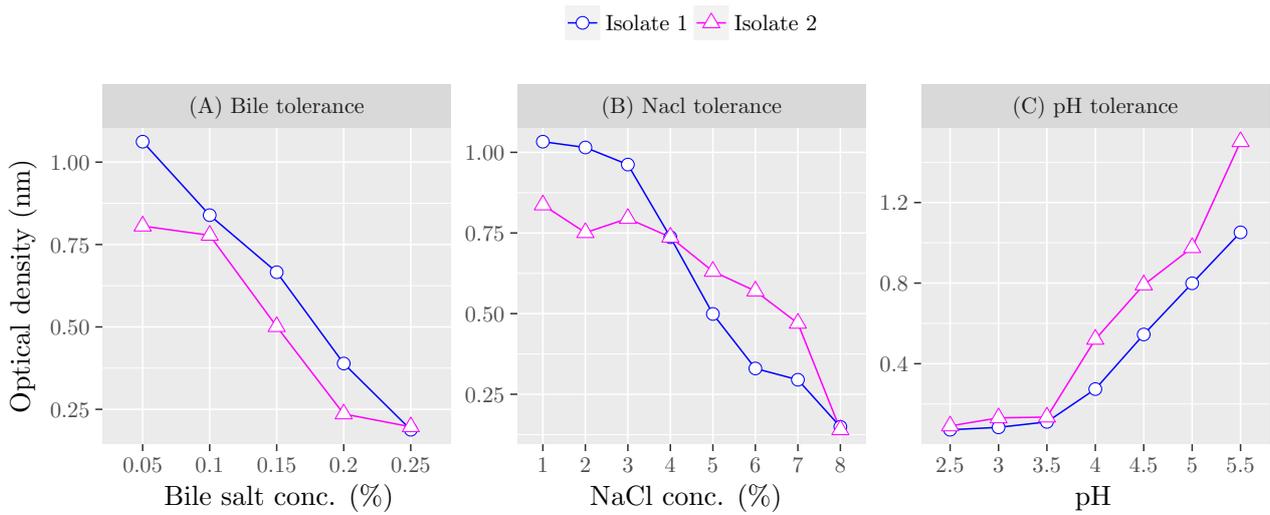


Figure 1. Tolerance of isolated *Lactobacillus* strains to bile and NaCl salt concentrations and change of pH

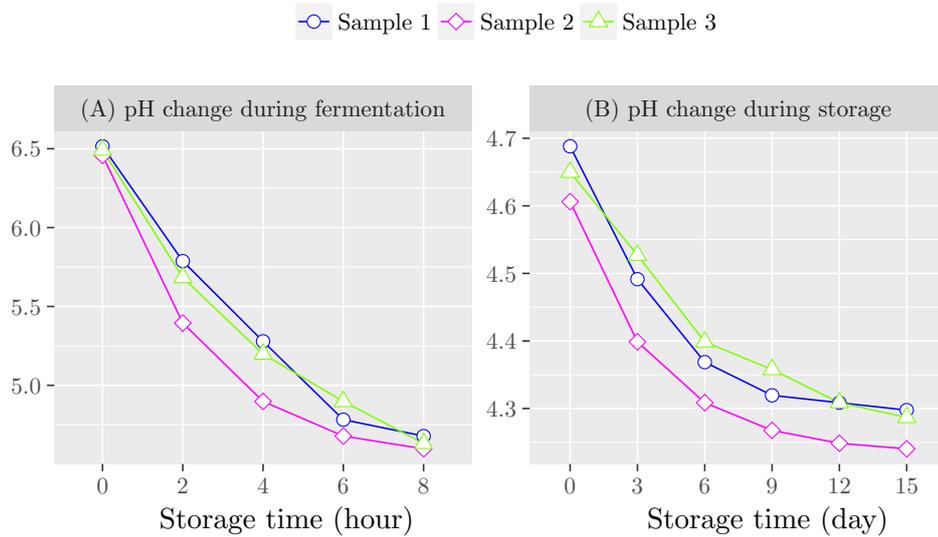


Figure 2. Tolerance of isolated *Lactobacillus* strains to bile and NaCl salt concentrations and change of pH

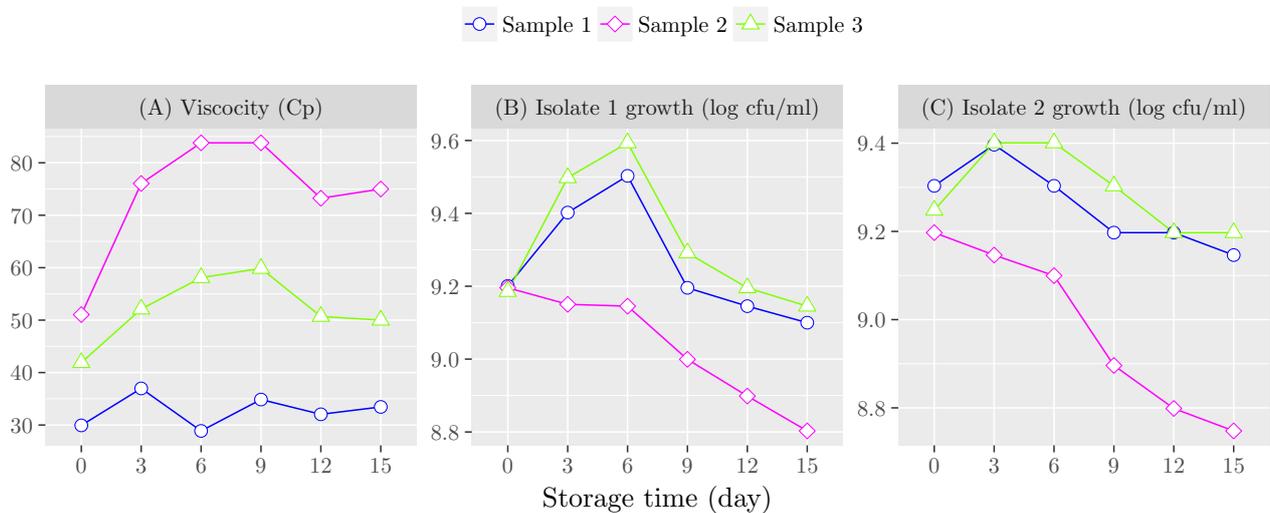


Figure 3. Tolerance of isolated *Lactobacillus* strains to bile and NaCl salt concentrations and change of pH

Table 1. Carbohydrate fermentation test of the *Lactobacillus* isolates

Sugar	Growth	
	Isolate 1	Isolate 2
Lactose	+	+
Dextrose	+	+
Maltose	-	+
Fructose	-	-
Sucrose	-	+

Table 2. Comparison of average chemical parameters of different types of probiotic milk drinks

Chemical parameters	Types of probiotics milk drinks		
	S ₁	S ₂	S ₃
Fat (%)	4.50±0.15	4.42±0.09	4.31±0.05
Protein (%)	3.99±0.17	3.67±0.12	3.45±0.12
Total solids (%)	22.31±0.15	24.61±0.14	26.73±0.15
Ash (%)	1.61±0.15	1.70±0.12	1.90±0.15
Acidity (%)	0.68±0.17	0.78±0.15	0.73±0.15
pH	4.69±0.12	4.61±0.12	4.65±0.15
Moisture content (%)	77.69±0.15	75.39±0.12	73.27±0.12

S₁ = Control, S₂ = Probiotic milk drinks mixed with 10% mango juice, and S₃ = Probiotic milk drinks mixed with 5% mango juice.

Table 3. ANOVA (Analysis of Variance) for overall acceptability

Sources of variance	Degree of freedom	Sum of squares	Mean squares	F-value	Probability
Judges	9	16.1	1.789	3.801	0.0083
Product	2	13.6	6.8	5.112	0.0058
Error	18	23.9	1.33		
Total	29	53.6			

Table 4. Duncan's Multiple Range Test (DMRT) for overall acceptability. LSD= 0.9624

Sample	Original order of means	Ranked order of sample	Ranked order of means
S ₁	6.10b	S ₂	7.10a
S ₂	7.10a	S ₃	6.70ab
S ₃	6.70ab	S ₁	6.10b

S₁ = Control, S₂ = Probiotic milk drinks mixed with 10% mango juice, and S₃ = Probiotic milk drinks mixed with 5% mango juice.

3.1.6 Bile salt test

The influences of bile salt of different concentration (0.05% to 0.25%) were observed among the two isolates namely Isolate 1 and Isolate 2. Based on the OD (optical density), it is evident that all isolates can grow from 0.05% to 0.25% as shown in Fig. 1 and therefore both can be used as probiotic.

3.1.7 NaCl tolerance test

The influences of NaCl of different concentration (1% to 8%) were observed among the two isolates namely Isolate 1 and Isolate 2. Based on the OD, it is evident that all isolates can grow from 1% to 8% as shown in Fig. 1 and therefore both can be used as probiotic.

3.1.8 pH tolerance test

The influences of culture pH (2.5 to 5.5) were observed among the two isolates namely Isolate 1 and Isolate 2. Based on the OD, it is evident that all isolates can grow from pH 2.5 to pH 5.5 as shown in Fig. 1 and therefore both isolates can be used as probiotic.

3.2 Development of probiotic milk drinks using isolated probiotic strain

Control and mixed fruit juice probiotic milk drinks samples after completion of fermentation were analyzed for fat, protein, total solids, acidity, pH and moisture content. The results are shown in Table 2.

It is seen that the highest fat content is given by S1 (4.50) and then followed in the order of S2 (4.42) and S3 (4.31). The highest protein content is given by S1 (3.99) and then followed in the order of S2 (3.67) and S3 (3.45). The highest ash content is given by S3 (1.90) and then followed in the order of S2 (1.70) and S1 (1.61). During fermentation of the probiotic milk drinks, it was found that the growth of Isolate 1 and Isolate 2 in pasteurized milk at 37 °C increased with increasing time. After 8 hr, the counts were higher than 9 cfu/ml, the pH was between 4.61-4.69 shown in Fig. 2 and the acidity was between 0.68-0.78. Therefore, the probiotic milk drink was ready and was chilled to slow down the fermentation and then stored at 5±1 °C.

During 15 days of storage of temperature 5±1 °C, the pH of the probiotic milk drinks of control and mixed with mango juice ranged between 4.2-4.3 shown in Fig. 2 and the acidity were ranged between 0.91 to 0.95%.

The viscosity of the probiotic milk drinks are shown in Fig. 3. The addition of mango juice into the probiotic milk drinks also affected the viscosity of the probiotic milk drinks as shown in Fig. 3. The increase of viscosity of probiotic milk drinks mixed with mango juice was due to the additional soluble

solids and it tended to decrease with increasing time as the cultures have grown and started to produce proteolytic enzymes (Shah, 2001).

From Fig. 3 it is clear that the counts of Isolate 1 and Isolate 2 during storage at 5±1 °C for 15 days ranged between 8.75 and 9.55 log cfu/ml. Lei and Jakobsen (2004) reported the number of probiotics which is greater than 5 log cfu/ml of the final product could be claimed to exert human health hence the probiotic milk drinks could be considered the health product as both isolate 1 and isolate 2 are more than 5 log cfu/ml.

The sensory evaluation showed that the scores of the 10% mango juice mixed with probiotic milk drink were significantly higher than that of the 5% and the liking score ranged in the medium-like (Table 3 and Table 4).

4 Conclusions

Obtained result suggested that, Isolate 1 and Isolate 2 during storage at 5±1 °C for 15 days ranged between 8.75 and 9.55 log cfu/ml those were higher than 5 log cfu/ml. That means isolated lactic acid bacteria (LAB) contain probiotic characteristics and among three samples, the probiotic milk drink that was mixed with 10% mango juice was the most acceptable.

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