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Genus and species-specific PCR revealed the presence of beneficial lactic acid bacteria in traditional Bangladeshi fermented milk products *Dahi*

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Abstract

This study aimed to identify beneficial lactic acid bacteria (LAB) from traditional Bangladeshi fermented milk product *dahi* and provide efficient starter cultures. Twenty *dahi* samples were collected from four districts in Bangladesh and selected isolates were then subjected to biochemical tests including catalase and gas production tests and physiological tests (growth under different conditions). Finally, LAB was identified at the genus and species level using PCR. Sixteen isolates were identified based on Gram-positive and catalase-negative characteristics. All of the isolates were homo-fermentative in nature. "Morphological and biochemical tests revealed that the majority of the isolates belonged to the genera Streptococcus (45.0%) and Lactobacillus (35.0% Physiological tests demonstrated that the most predominant *Streptococci* were *Streptococcus thermophilus* which exhibited profuse growth at 45°C and a pH of 4-5, but no growth at below 15°C and even in 2% NaCl. The other strains were *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* which manifested luxuriant growth at 45°C, moderate growth at 25°C and 2% NaCl and no growth below 15°C. Furthermore, genus-specific PCR followed by species-specific PCR also verified the presence of these species as 25% of the 16 isolates were identified as *S. thermophilus*, 31.2% as *L. delbrueckii* ssp. bulgaricus and 12.5% as L. acidophilus with these methods. Positive PCR results demonstrated that among the isolated strains, 25% of LAB were *S. thermophilus,* 31.2% were *L. bulgaricus* and 12.5% were L. *acidophilus*. These identified bacteria are the most widely used LAB in many fermented milk products such as dahi, yogurt and yogurt-like products. Therefore, they can be used as starter cultures to increase the probiotic and organoleptic properties of dahi and other Bangladeshi fermented milk products.

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Introduction

Dahi is a popular dessert in Bangladesh and other South Asian countries that has a high nutritional value and assists in digestion (Harun-Ur-Rashid *et al.*, (2006; Shangpliang *et al.*, 2017). It is made by traditional and natural fermentation of milk by adding previously produced dahi containing various lactic acid bacteria (LAB) and/or different bacterial fermentation cultures. Briefly, boiled milk is mixed with *dahi* samples made earlier and kept for natural spontaneous fermentation at room temperature for 2–3 days. It is mostly produced from cow milk and to a lesser extent from buffalo milk and has several similar characteristics like yogurt. Because of the presence of LAB, *dahi* possesses antimicrobial and probiotic properties such as stabilization of gut microbiota, palliation of lactose intolerance, reduction of serum cholesterol and elicitation of the immune system and antitumor activity (Isolauri *et al.*, 2001; Østlie *et al.*, 2003; Taye *et al.*, 2021). Numerous studies found that LAB can inhibit the growth of numerous food-borne pathogens and prevent food spoilage by rapidly producing various compounds with antimicrobial activities, such as lactic and acetic acid, acetaldehyde, ethyl alcohol and various antimicrobial toxins, including reuterin and reutericyclin (Adeniyi *et al.*, 2015; D. Ren *et al.*, 2018).

Lactic acid bacteria are predominantly found in many fermented foods including, yogurt, curd, and cheese and are reported in many ethnic fermented products in Asia, Africa and the Middle East (Burentegusi *et al.*, 2002; Mathara *et al.*, 2004; Md. Ibrahim Khalil & Md. Nural Anwar, 2016). LAB received considerable importance due to having probiotic characteristics and maintaining the optimum environment in the gut by reducing gastrointestinal pathogenic microorganisms including *Clostridium difficle*, *Helicobacter pylori* and rotavirus (Ljungh & LastName, 2006). Several bacteria such as *Lactobacillus*, *Streptococcus* and *Enterococcus* have been found to be active candidates as probiotics for human and animal consumption (Vantsawa *et al.*, 2017). Additionally, LAB can degrade proteins and lipids and are used in alcohol, acid and ester production which contribute to the production and development of specific flavors in fermented foods (Schrader, 2007). Typically, LAB are Gram-positive with certain distinguishing characteristics such as non-respiring, non-spore-forming and typically rod or cocci. Their optimum growth is seen at a slightly lower acidic state (pH 5.5 – 6.0) and is predominantly fermentative (Md. Ibrahim Khalil & Md. Nural Anwar, 2016). LAB can metabolize carbohydrates via homolactic fermentation where glucose is converted into lactic acid or heterolactic fermentation where ethanol and CO2 are the end products ("The Transformation of Must into Wine," 2012).

However, as discussed previously, *dahi* is a naturally fermented milk product. Unlike many fermented milk products in developed countries, the availability of dried starter culture in Bangladesh is scarce. Therefore, producers largely rely on previously produced *dah*i or marketed yogurt or bacterial cultures that are not well-characterized and often have a low viability. Generally, the quality of any fermented milk is largely attributed to the quality of bacterial cultures.

The Food and Drug Administration (FDA) recommended several LAB such as *Lactococcus lactis, Bifidobacterium* species, and *Leuconostoc* species are used for starter culture in addition to *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Freitas, 2017). Hassan *et al.* revealed that bacterial cultures used for fermentation in Bangladesh were not of satisfactory quality because of adulterations and unhygienic conditions in stores. Moreover, the fermented yogurt or *dahi* is usually preserved for a long time in freezing conditions which may vitiate its quality and disrupt the viability of LAB. Traditionally, isolation and characterization of LAB have been done by plate counting methods employing selective media and biochemical tests (Burentegusi *et al.*, 2002; Harun-Ur-Rashid *et al.*, 2006). However, the recent technological advances in molecular genetics have allowed scientists to determine *Lactobacilli* species more accurately. Particularly, developments in 16S rDNA sequencing as well as genus and species-specific PCR have made differentiation of bacterial species based on their

known 16S rDNA gene much more reliable and easier (Dhameliya *et al.*, 2020; Dubernet *et al.*, 2002; Y. Ren *et al.*, 2015).

Considering the above-mentioned issues, this study aims to isolate and identify the most widely used and beneficial lactic acid bacteria that exist in traditional Bangladeshi fermented milk product *dahi* samples by biochemical and molecular techniques using genus and species-specific PCR of 16S rDNA. This may provide more effective starter cultures to produce a higher quality *dahi* and preserve this traditional fermented milk product by maintaining the microbial cultures.

Materials and methods

Sample collection, preparation and maintenance

Twenty *dahi* samples were collected from local shops in four districts (Rajshahi, Bogura, Bhola, and Khulna) in Bangladesh. The areas were selected to assess the starter cultures and *dahi* of different regions of the country. The samples were preserved using a thermal flask and an icebox maintaining a temperature of 4°C –5°C and stored in a refrigerator temperature for 24 hours before analysis. Then 10mL of dahi samples were homogenized with 90mL of 0.85% (w/v) NaCl solution and mixed thoroughly to prepare the initial dilution. Two culture media were used to enumerate viable LAB: deMan, Rogosa and Sharp (MRS) agar (Merck, Germany) and M17 agar (Merck, Germany). After preparing a tenfold serial dilution, 0.1mL aliquots were dispersed onto MRS and M17 agar. MRS agar plates were incubated at 37°C while M17 plates were incubated at 32°C for 48 hours. Afterwards the number of visible colonies was counted and the number of colony-forming units (cfu) /mL of sample was indicated (Md. Ibrahim Khalil & Md. Nural Anwar, 2016).

Isolation and identification of LAB through morphological, biochemical and physiological tests

Gram's Staining and catalase tests

The colonies with distinguished morphology, color and shape were considered LAB and isolated to be purified by streaking onto BCP agar followed by subculturing in TYLG broth as described by Harun *et al.* (2007) (Harun-Ur-Rashid *et al.*, 2006). Bacteria that stained blue-purple were Gram-positive, while bacteria that stained pink-red were Gram-negative (Kefir *et al.*, 2018). Catalase test was performed to observe the catalase enzyme production by LAB. Briefly, bacterial culture was picked aseptically with a sterile loopful and mixed with a drop of 3% H_2O_2 on a clean microscopic slide.

Gas production tests

The fermentation nature (homo- or heterofermentative) of gram-positive and catalase-negative isolates was determined by the production of CO2 from glucose. Bacterial cultures activated overnight (50 µl) were suspended into 8mL MRS broth that contained an inverted Durham tube. The broth was incubated at 37°C for 5 days, after which the possible accumulation of CO2 in the tube was observed.

Growth at different temperatures, NaCl concentrations and pH

LAB were exposed to different growth conditions such as temperatures, sodium chloride concentrations and acidic environments. To study different temperatures, overnight activated bacterial cultures (50 µl) were transferred into 5mL MRS broth containing 0.004% bromecresol purple and incubated at 10°C, 15°C, 25°C and 45°C for 48 h and 72 h. To determine their growth at different sodium chloride concentrations, 50µl of bacterial cultures was inoculated into MRS broth containing 4.95 g of 2%, 4% and 6.5% NaCl solutions and 0.004% bromecresol purple. The growth of overnight activated bacteria culture was spread into MRS broth and determined at pH 3.0, 4.0, 4.5, 6.5, 8.0 and 8.5. Strains of LAB were identified via color changes from purple to yellow in different conditions.

Molecular characterization of LAB using genus and species-specific primers

Isolation of Genomic DNA

Genomic DNA of gram-positive and catalase-negative isolates was extracted according to the instructor's manual (Invitrogen, USA) cultivating them in MRS broth at 30°C for 48-72 hours (Tilahun *et al.*, 2018). DNA was quantified by a NanoDrop one spectrophotometer (ThermoFisher Scientific, USA) and visualized using gel documentation.

Identification of LAB by genus-specific PCR

The detection of the *Lactobacillus* genus was performed according tomcorist *et al* (McOrist *et al*., 2002). or this, the target region of the extracted DNA was amplified using the primers indicated in Table 1. PCR was conducted in a thermal cycler (Applied Biosystems, USA). The reaction conditions for PCR were as follows: an initial denaturation at 94°C for 4 minutes followed by 30 cycles of 94°C for 15 seconds, 57°C for 15 seconds for annealing, 72°C for 15 seconds for extension and 72°C for 4 minutes for final elongation. The identification of the Streptococcus genus was performed according to De-Xaxars *et al* (Mas-De-Xaxars & Garcia-Gil, 2009). The target region was amplified by the primers shown in Table 1. The reaction conditions were as follows: an initial denaturation at 95°C for 10 minutes followed by 30 cycles of 94°C for 30 seconds, 67°C for 1 minute for annealing, 72°C for 1 minute for extension and 72°C for 10 minutes for final elongation.

Identification of LAB by species-specific PCR

Isolates belonging to the genus Lactobacillus or Streptococcus were subjected to a further PCR to differentiate between Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus. Therefore, the method described by Tabasco *et al*. (2000) was applied (Tabasco *et al*., 2007). The used primers are indicated in Table 1 and the PCR conditions were as follows: an initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 20 seconds for annealing, 72°C for 20 seconds for extension and 72°C for 5 minutes for final elongation.

Table 1. List of genus and species-specific primer.

Name of bacteria	Name	DNA Sequence 5′-3′	Product size	References
Lactobacillus spp.	For	TGGAAACAGGTGCTAATACCG	247 bp	(McOrist *et al*., 2002)
	Rev	CCATTGTGGAAGATTCCC		
Lactobacillus. acidophilus	For	AGCGAGCTGAACCAACAGAT	227 bp	(Tabasco *et al*., 2007)
	Rev	AGGCCGTTACCCTACCAACT		
Lactobacillus bulgaricus	For	TCAAAGATTCCTTCGGGATG	232 bp	(Tabasco *et al*., 2007)
	Rev	TACGCATCATTGCCTTGGTA		
Streptococcus spp.	1043F	CACTCTAGCGAGACTGCCG	450 bp	(Mas-De-Xaxars & Garcia-Gil, 2009)
	1492R	ACGGTTACCTTGTTACGACTT		
Streptococcus. thermophiles	For	ACGCTGAAGAGAGGAGCTTG	157 bp	(Tabasco *et al*., 2007)
	Rev	GCAATTGCCCCTTTCAAATA		

Result and discussion

Isolation and enumeration of lactic acid bacteria

Fig. 1 illustrates the cfu of LAB populations in 1mL of different dahi samples. It was observed that a large number of LAB was present in the samples and ranged from 1.4×10^8 cfu/ml to 2.4×10^9 cfu/ml in MRS media and 3×10^7 cfu/ml to 2.8×10^9 cfu/ml in M17 media. More than 50% of all samples demonstrated 5×10^8 cfu/ml LAB. Higher bacterial counts (>25.0×10^8 cfu/ml) were observed in S10 and S11 samples from Bhola and S19 and S20 samples from Rajshahi, while lower bacterial counts (<10.0×10^7 cfu/ml) were observed in S22 and S23 *dahi* samples from Khulna in M17 agar media. On the contrary, only the S19 yogurt sample from Rajshahi showed a higher bacterial count (2.4×10^9 cfu/ml) in MRS media and the other samples in MRS media exhibited moderate LAB counts. The study samples contained a higher bacterial count compared to other studies. Amanullah *et al*. found that LAB ranged from 1.0×10^4 to 9.5×10^5 cfu/ml in the samples (Amanullah *et al*., 2020). Khalil *et al*. revealed that viable bacteria was higher in anaerobic condition (6.1×10^5 - 5.4×10^8) cfu/ml and lower in aerobic condition (1.0×10^5 - 5.6×10^7) compared to our findings (Md. Ibrahim Khalil & Md. Nural Anwar, 2016). The variations in the LAB counts may be due to the differences in carbon sources and the utilization and digestion of the bacteria that were present (Gänzle & Follador, 2012; Hayek *et al*., 2013). It was observed that colony formation was more prevalent in the M17 media compared to MRS media. MRS media is well-defined

media for most of the LAB. However, *Streptococci* demonstrate a weak growth in that media and hence another media was developed known as M17 (Hayek *et al.*, 2019). Our subsequent analysis revealed that *Streptococci* was the most common genus in the study samples.

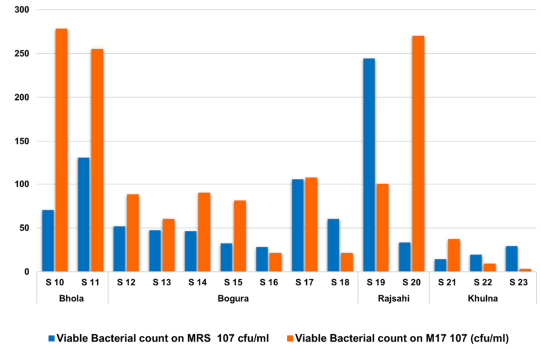

Fig. 1. Enumeration of viable bacterial from MRS and M17 agar media.

Determination of genus by morphological, biochemical and molecular test

Sixteen colonies were isolated as lactic acid bacteria from MRS and M17 media of *dahi* samples based on Gram staining and catalase reaction. Morphological studies of the samples demonstrated that the majority of the colonies were either cocci-shaped (45.0%) or rod-shaped (35.0%) which were subjected to biochemical and molecular characterization. The rod-shaped, distinct white or whitish yellow-colored bacteria represent the *Lactobacillus* genus while the cocci-shaped, flat or circular, grayish-colored bacteria represent the *Streptococcus* genus (Hutkins, 2006). Besides, all of the isolated strains were gram-positive and catalase-negative. Catalase enzyme causes the decomposition of hydrogen peroxide into oxygen and water. The production of O_2 was observed by the production of O_2 bubbles on the slides and indicated the catalase-positive character of isolated bacteria (Azadnia & Khan Nazer, 2009). Gas production test was performed to observe the fermentative nature of the LAB. It was observed that all the isolates fermented glucose and produced only lactic acid and thus were homofermentative(Hutkins, 2006). Table 2 depicts the morphological and biochemical characteristics of isolated colonies. This finding is in line with the findings of Amanullah *et al.* (2020) and Azadnia *et al.* (2009) (Amanullah *et al.*, 2020; Azadnia & Khan Nazer, 2009). Additionally, gel documentation followed by genus-specific PCR revealed that the seven isolates were *Lactobacillus* genus (247 bp) while nine isolates were *Streptococcus* genus (560 bp). The findings are supported by previous investigations. Islam *et al.* revealed that *Streptococcus* (50.82%) and *Lactobacillus* (39.92%) were the predominant bacteria present in almost all types of yogurts in Bangladesh (Islam *et al.*, 2021). Another study also found that the most prevalent LAB in Bangladeshi yogurt were *Streptococcus* (50%) and *Lactobacillus* (27%) (Harun-Ur-Rashid *et al.*, 2006). However, Amanullah *et al* showed in their study that *Lactobacillus* and *Leuconostoc* genus were predominant in Bangladeshi yogurt (Amanullah *et al.*, 2020).

Table 2. Morphological and biochemical test results of isolated colonies.

Source	Sample Number	Catalase test	Gram's staining	Shape	Gas production test
Bhola	S10	-	+	Rod	Homo
	S11	-	+	Rod	Homo
	S11	-	+	Cocci	Homo
Bogura	S12	-	+	Cocci	Homo
	S13	-	+	Cocci	Homo
	S14	-	+	Rod	Homo
	S15	-	+	Cocci	Homo
	S16	-	+	Cocci	Homo
	S17	-	+	Cocci	Homo
	S18	-	+	Rod	Homo
Rajshahi	S19	-	+	Rod	Homo
	S19	-	+	Cocci	Homo
	S20	-	+	Cocci	Homo
Khulna	S21	-	+	Rod	Homo
	S22	-	+	Cocci	Homo
	S23	-	+	Rod	Homo

Determination of species by physiological and molecular test

Table 3 delineates the physiological characteristics of the isolated LAB. Physiological tests showed that four (25%) of the nine isolates of the *Streptococcus* genus were *Streptococcus thermophilus*. It was a thermophilic LAB that exhibited profuse growth at 45°C, but no growth at below 15°C and even in 2% NaCl. They could grow even at a pH of 9.5 but optimum growth was at 4-5. Furthermore, species-specific PCR identification of the *Streptococcus* genus found the presence of *S. thermophilus*.

The other five species were not identified by species-specific PCR, but their features represented that of *S. bovis* which may, according to Harun-ur-Rashid *et al.*, contributes to the quality and characteristics of *dahi* (Harun-Ur-Rashid *et al.*, 2006).

In addition to this, five isolates (31.2%) among the 16 samples were identified as *Lactobacillus delbrueckii* subsp. *bulgaricus*. These demonstrated luxuriant growth at 45°C, moderate growth at 25°C and no growth below 15°C. Moreover, they can only tolerate 2% NaCl while optimum growth was observed at a pH of 4-5. The other two isolates of *Lactobacillus* also demonstrated similar results in biochemical and physiological tests. However, species-specific PCR revealed these as *Lactobacillus acidophilus*. This result is similar to Mithun *et al.* (2015) (Mithun *et al.*, 2015) and Abdullah *et al.* (2010) (Abdullah & Osman, 2010). Molecular identification by species-specific PCR clinched the abovementioned results (Fig. 2 and Fig. 3).

Our findings differ from previously published studies on Bangladeshi yogurt or *dahi*. While Harun-ur-Rashid *et al.* delineated that *S. bovis* and *L. fermentum* were the predominant LAB present in *dahi* (Harun-Ur-Rashid *et al.*, 2006), Amanullah *et al* found that *Leuc. cremoris* and *L. lactisin* were the most common species (Amanullah *et al.*, 2020). However, the strength of our study is the identification of genus and species of lactic acid bacteria by 16S rDNA-specific PCR.

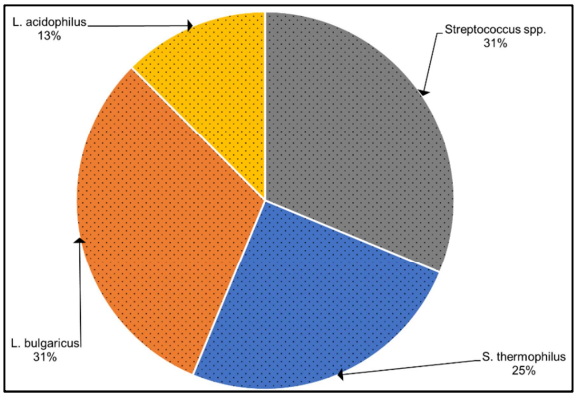

Fig. 2. Distribution of lactic acid bacteria at the species level.

Table 3. Physiological test result of isolated colonies.

Isolates	Bhola				Bogura						Rajshahi			Khulna		
	S10	S11	S11	S12	S13	S14	S15	S16	S17	S18	S19	S19	S20	S21	S22	S23
Growth at temperature (°C)																
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Growth in NaCl (%)																
2.0	+	+	-	-	-	+	-	-	-	+	+	-	-	+	-	+
4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH																
2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
5.0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9.5	-	-	+	+	+	-	+	+	+	-	-	+	+	-	+	-

The DNA quantification results demonstrated that the isolated genomic DNA was intact and the average concentration of isolated DNA was 159.14 ng/µl. The average ratio of absorbance at 260/280 was 2.63 and at 260/230 was 2.79 which indicates high quantities of DNA with low purity.

PCR amplification of 16S rDNA of the selected LAB demonstrated that the size of the amplified product was 247 bp for *Lactobacillus* spp., 227 bp for *L. acidophilus* (Fig. 3A), 232 bp for *L. bulgaricus* (Fig. 3B), 450 bp for *Streptococcus spp.* and 157 bp for *S. thermophiles* (Fig. 3C).

Our findings are supported by previous studies bymcorist *et al.* De-Xaxars *et al.* Tabasco *et al.* (Mas-De-Xaxars & Garcia-Gil, 2009;mcOrist *et al.*, 2002; Tabasco *et al.*, 2007) where they used genus and species-specific PCR of 16S rDNA to identify lactic acid bacteria from different samples including fermented milk. Identification by 16S rRNA or 16s rDNA sequencing has become a standard technique for determining relationships among bacteria and identifying important bacteria. However, there are certain drawbacks of 16s sequencing such as variation in PCR amplicons, high costs and experimental limitations. Additionally, most of the sequence studies can accurately determine the genus level and not the species level due to limited sequence variations. Identification of LAB species is very crucial for complying with the regulations and specifications of fermented products, labeling appropriately and claiming the health benefits of probiotic LAB. Subsequently, polymerase chain reaction (PCR) of 16S rDNA by genus and species-specific primers have more prospects in bacterial identification and received widespread attention. Moreover, combination of PCR and other tools such as denaturing gradient gel electrophoresis (DGGE) has a greater advantage of identifying bacteria with more selectivity and specificity (Tabasco *et al.*, 2007). However, precise detection and identification of bacteria depends on many factors such as primers, PCR conditions and samples.

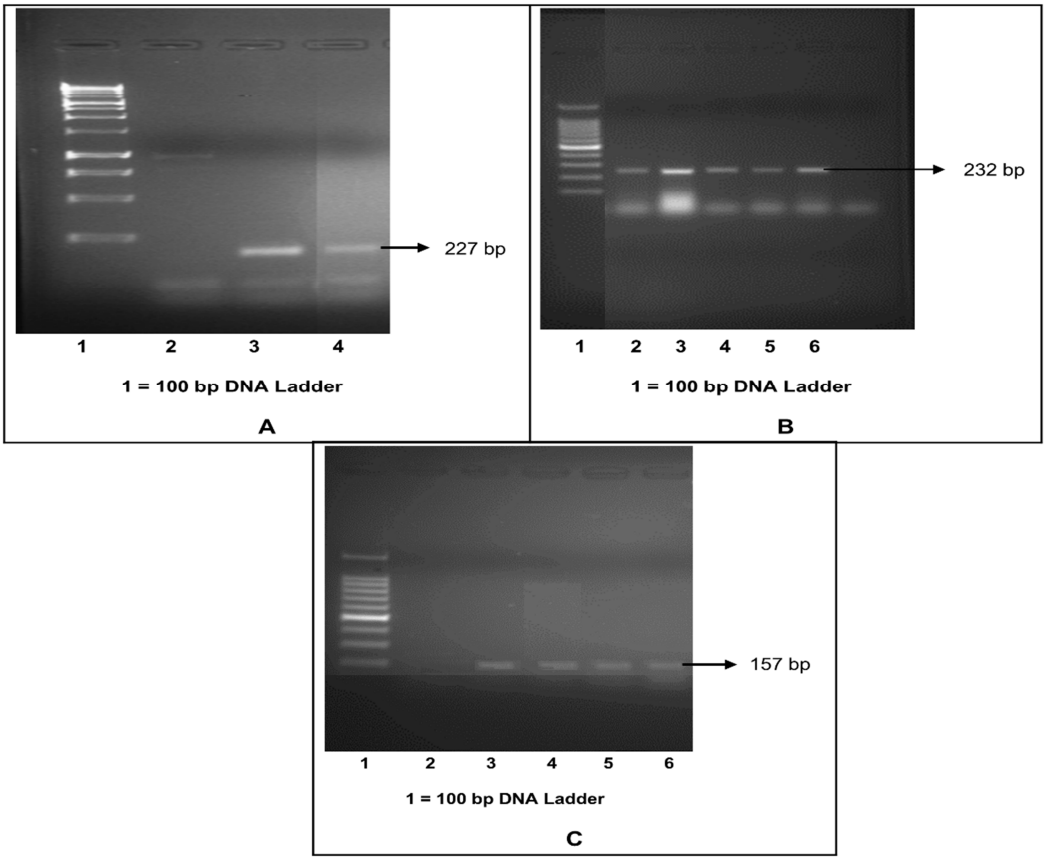

Fig. 3. Species-specific PCR product of (A) *Lactobacillus acidophilus*, (B) *Lactobacillus delbrueckii* sub sp. *Bulgaricus* and (C) *S. thermophiles*.

All the identified strains in *dahi* samples have extensive probiotic potential that provides immunity against pathogenic microbes, increases vitamin and mineral metabolism, reduces serum cholesterol and blood glucose level and aids against food allergies (Freitas, 2017). L. acidophilus produces antibiotics along with lactic acid and H_2O_2 and aid in nutrient utilization such as protein, calcium and iron (Litopoulou-Tzanetaki & Tzanetakis, 2014). *S. thermophilus* and *L. bulgaricus* were also found to be associated with the reduction of cholesterol. The FDA has characterized yogurt which is much similar to

dahi as fermentation milk produced by fermentative LAB *S. thermophilus* and *L. bulgaricus* (Freitas, 2017). Commonly, these two thermophilic LAB species facilitate each other growth by a process termed 'protocooperation'; *L. bulgaricus* produces aromatic compound formate that stimulates the growth of *S. thermophilus* while amino acid produced by *S. thermophilus* stimulates the growth of *L. bulgaricus* (Stanley, 2003). This interaction creates a positive effect on dairy products such as increasing organoleptic properties and numerous health benefits (Chen *et al.*, 2017). A clinical study showed that children who consumed yogurt fermented with these bacteria had significantly less persistent diarrhea than children who consumed milk (Boudraa *et al.*, 1990).

Conclusion

It was observed in the study that most of the lactic acid bacteria present in traditional Bangladeshi milk-*dahi* were grouped into *Streptococcus* and *Lactobacillus* genus. Moreover, molecular identification by genus and species-specific PCR of 16S rDNA of the LAB revealed that *S. thermophilus*, *L. bulgaricus* and *L. acidophilus* were most prevalent. These LAB are being used in dairy industries and at home for the production of yogurt and yogurt-like products including *dahi*. Their presence significantly improves the sensory and probiotic potential of *dahi* and these can be used as starter cultures for producing high-quality *dahi* and other Bangladeshi fermented milk products which will ultimately benefit the small-scale dairy industries of the country.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

All the authors declared that they have

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Author contributions

Conceptualization and Methodology: MAK and SMA; Investigation: MAK, SMA, DD, SFS and AAN; Resources and Data Curation: MAK, SMA and SFS; Formal analysis: MAK, AAN, and SFS Writing—original draft preparation, AAN, MAK, DD and SFS; Writing—review and editing, AAN, MAK, SMA and SMJ; Supervision, MAK, SMA and SMJ; Project Administration, MAK. All authors have read and agreed to the published version of the manuscript.

Abbreviations

LAB: Lactic acid bacteria; PCR: polymerase chain reaction; FDA: Food and Drug Administration

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