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Original Article

Artisanal buffalo milk curd from *Charfassion* upazila of *Bhola* district in Bangladesh as a potent source of bifidobacteria

M. U. Habiba[1], A. A. Jhinuk[1], S. M. R. Sumon[2], M. Jahan[3], S. Ahmed[1], M. D. Hossain[4], A. S. M. Selim[4] and M. M. Rahman[1]*

[1]Department of Dairy and Poultry Science, Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh
[2]Department of Medicine, Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh
[3]Department of Microbiology and Public Health, Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh
[4]Department of Animal Science and Nutrition, Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh

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***Corresponding Author**

M. M. Rahman, E-mail: morshed@bsmrau.edu.bd

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A B S T R A C T

The present study explored the traditionally prepared fermented buffalo milk curd to isolate and characterize *Bifidobacterium* sp. towards developing future probiotics. Standard methods have been applied to identify *Bifidobacterium* sp. using bifidobacteria selective media, bifidobacteria specific mupirocin resistance test, phosphoketolase activity and commercial biochemical kit. The strains further confirmed by *xfp* gene PCR amplification. The growth dynamics of the identified isolates was also determined by monitoring the turbidity of the cultured media spectrophotometrically. A total of 20 isolates obtained from selective media were found gram positive, catalase negative, rod or v-shaped, non-motile and identified as presumptive bifidobacteria. All the isolates were resistant (zone diameter <10 mm) to the antibiotic mupirocin. However, the phosphoketolase activity of the isolates were varied significantly (p<0.05). A high, moderate and weak/no phosphoketolase activity was exhibited by 35%, 20% and 45% isolates, respectively. In the contrary, fourteen isolates (70%) amplified well with phosphoketolase activity encoded *xfp* gene and produced a PCR product of 704 bp. Combining the findings of these three tests, only six isolates (30%) displayed mupirocin resistance, high phosphoketolase activity and amplification with *xfp* gene. Of these six isolates biochemical test confirmed three isolates as *Bifidobacterium mongoliense* and two as *Bifidobacterium pseudolongum* subsp. *globosum*. The exponential phase growth rate of the six isolates also varied significantly (p<0.05) and ranged from 0.16±0.01/h to 0.34±0.02/h. Traditionally prepared buffalo milk curd from *Charfassion* upazila of *Bhola* district, Bangladesh could be a potent source of *Bifidobacterium* sp. However, species-specific identification and elucidation of probiotic potential are pivotal for future industrial use.

Introduction

Traditional fermented milk products are common in rural communities all over the world as it is an inexpensive and easy way to conserve milk (Jatmiko *et al*., 2018). *Dahi* is considered as one of the oldest fermented milk products throughout the Indian sub-continent and often considered as analogue to the western fermented product yogurt (Sarkar, 2008). According to Ayurveda, a pseudoscientific alternative medicine system in the Indian sub-continent, *dahi* is recommended for alleviating of many gastrointestinal disorders such as dysentery, dyspepsia (Gandhi and Natrajan, 2014). Fresh or boiled milk (cow, buffalo or mixed species) are being fermented naturally or with previous day's *dahi* (a process called back slopping) to prepare traditional *dahi*

(Verma *et al*., 2012). In Bangladesh *dahi* is extensively prepared using traditional back slopping method except at industry level. Buffalo milk curd is one of the traditional fermented dairy products found in *Bhola* district, a largest island in southern-central of Bangladesh. The product is prepared from raw buffalo milk (without processing and/or boiling) that indicates the local manufacturers' relies on the natural microbiota of milk. Moreover, the milk used to prepare these traditional dairy products comes from those dairy buffaloes mainly fed on natural grazing. Therefore, the possibility of microbial diversity in these products is very high. According to the scientific report, traditional dairy products especially those made from raw milk are thought to have microbial diversity and are considered as natural sources of potential probiotic bacteria (Landry *et al*., 2017).

Bifidobacteria are the predominant bacteria of infant intestine (Scardovi, 1986). In 1899, bifidobacteria was first isolated from the feces of breast-fed infants and since then they have been isolated from diverse ecological niches. The members of the genus *Bifidobacterium* have been included as live components in a variety of functional foods (Ventura *et al*., 2004). They are helpful in maintaining a proper balance in the human intestinal flora, playing a protective role against potential pathogens and putrefactive bacteria, hence they have been included in the probiotics group (Biavati *et al*., 2000). There are many other health aspects related to the use of *Bifidobacterium* sp. such as treatment and prevention of diarrhea, reduction in lactose intolerance in some individuals, increase resistance to microbial infections, potential role in cancer prevention, possible treatment of inflammatory bowel disease, alleviation of constipation, impact on immune function and reduction of serum cholesterol (Leahy *et al*., 2005). In addition, they have been using as probiotics for preventive and therapeutic purposes in newborns and infants considering their high abundance in the gastrointestinal tract, their capability of colonizing the gut, and their long history of safe use (Sanders *et al*., 2010). Bifidobacteria have been using in a wide variety of probiotic dairy products including fermented milk, cheese, yogurt and ice cream. However, the survival of bifidobacteria in fermented dairy products depends on several factors such as the strain of bacteria used, fermentation conditions, storage temperature, and preservation methods. The growth of bifidobacteria in milk is often slow or limited compared with lactic acid bacteria used in fermented dairy products, and this appears partially due to low proteolytic activities (Roy, 2005). Therefore, novel strains of bifidobacteria with diverse functional properties are always of great interest.

Bifidobacteria has been isolated from various sources like human gut, feces, breast milk, milk and fermented milk, sewage, chicken, rat, rabbit, infant, piglet, chimpanzee, honeybee (Baffoni *et al*., 2013); however, few from traditional fermented dairy products like *Bifidobacterium* from Croatian Cheese (Croatian raw ewe's milk cheeses), *Bifidobacterium crudilactis and B. mongoliense* from French raw milk cheese (Delcenserie *et al*., 2013), *Bifidobacterium longum* from raw camel milk (Yasmin *et al*., 2020), *B. mongoliense* from airag (Watanabe *et al*., 2009), oscypek, water kefir (Laureys *et al*., 2016). It is noteworthy that isolation of bifidobacteria from fermented food products could be considered as safe as they have already been using for human consumption. In Bangladesh, 40% of the total buffalo population (1.457 million heads) are raised in coastal areas (Hamid *et al*., 2016) resulted availability of buffalo milk in these areas. The *Bhola* district in particular the *Charfassion* upazila and the adjacent areas are considered as the origin of raw buffalo milk curd from the ancient time. However, few other coastal areas are also known to produce such product. However, no attempt has been taken to isolate *Bifidobacterium* species from natural resources or fermented dairy products in Bangladesh. So far, this was the first study of its kind in Bangladesh to isolate bifidobacteria from a naturally fermented traditional dairy product. Taken together, the present research was designed for the isolation and biochemical as well as genus specific identification of *Bifidobacterium* species from traditional buffalo milk curd.

Materials and Method

Collection of curd samples

The *Charfassion* upazila of *Bhola* district is one of the famous places to produce raw buffalo milk curd. The local curd manufacturers' of the upazila collected raw milk from the buffalo farmers (called *Bathan*) of various *Char* areas like *Ranga Bali*, *Monpura*, *Dhal Char*, *Char Montaj*, *Char Kukri-mukri*. After preparation, curd samples from four different manufacturers' were collected and transported to the laboratory of Dairy and Poultry Science department, Bangabandhu Sheikh Mujibur Rahman Agricultural University maintaining refrigerated temperature in ice box containing ice pack.

Isolation of presumptive bifidobacteria

The presumptive bifidobacteria were isolated from the four buffalo milk curd samples using Bifidobacteria Selective Count Agar Base (HiMedia Laboratories Pvt. Ltd., India) supplemented with mupirocin (50 mg/L) and 1% glacial acetic acid (10 ml/L). After homogenization, each curd sample was serially diluted (from 10^{-1} to 10^{-6}) with phosphate buffered saline (PBS; pH 7.2) and sample from each dilution was evenly spread onto the agar plates in duplicate. The inoculated plates were incubated under anaerobic condition (anaero-gas pack system includes anaero jar and gas pack, HiMedia Laboratories Pvt. Ltd., India) at 37°C for 48 hours. After incubation, at least five colonies from the high dilution plates for each sample were randomly selected. The selected colonies were re-purified by subsequent subcultures in the same media. The purified colonies were characterized using Gram stain, cell morphology, catalase reaction and motility tests. Gram-positive, rod shaped, non-motile and catalase-negative isolates were preserved at -86°C in *Lactobacillus* MRS broth (HiMedia Laboratories Pvt. Ltd., India) supplemented with 0.05% L-cysteine.HCl and 30% (v/v) glycerol for further analysis.

Mupirocin resistance test

The antimicrobial susceptibility to mupirocin was performed by the agar disc diffusion method as described by Domingos-Lopes *et al*. (2017). After preparing, MRS agar plate supplemented with 0.05% L-cysteine.HCl was inoculated with the respective isolate by swab technique. Prior to swabbing, each isolate was grown in MRS broth containing 0.05% L-cysteine.HCl anaerobically at 37°C. The grown cells were re-suspended in sterilized saline solution (0.85% NaCl) to an optical density of 1.0 at 660 nm (equivalent to McFarland standard 0.5). After evenly distributing the inoculum, the 5 µg mupirocin disc (HiMedia Laboratories Pvt. Ltd., India) was then placed onto the surface of the respective plate and incubated anaerobically at 37°C. After 24h incubation, the diameter of the inhibition zone around

the disc was measured using a caliper. The measured inhibitory zone ≤10 mm was considered as resistant to mupirocin.

Phosphoketolase assay

The phosphoketolase assay was carried out according to the protocol described by Orban and Patterson (2000). Each isolate was reactivated anaerobically by subculturing at least three times in MRS broth supplemented with 0.05% L-cysteine.HCl at 37°C. Cells were obtained from ten ml of overnight grown respective bacterial culture by washing twice (10,000×*g*, 4°C, 15 min) with phosphate buffer (0.05 M KH_2PO_4 and 0.05% L-cysteine.HCl mixed 1:1 (V/V), adjusted to pH 6.5) and resuspended in 1.0 ml of the same buffer. Washed bacterial cells were incubated with 0.4ml (450 mg/ml stock solution) of hexadecyltrimethylammonium bromide (cetrimonium bromide, CTAB) for 5 min prior to the assay. After pretreatment, 0.25 ml of sodium fluoride (NaF, 3 mg/ml) and sodium iodoacetate (5 mg/ml) solution in H_2O was added followed by the addition of another 0.25 ml sodium fructose-6-phosphate (80 mg/ml in H_2O). The solution was vortexed and then incubated at 37^{o}C for 30 min. After incubation, 1.5 ml of hydroxylamine. HCl (13 g/100 ml) was added, vortexed and allowed to incubate at room temperature for 10 min. One milliliter of TCA (15%, W/V), 1.0 ml of 4N HCl and 1.0 ml of ferric chloride ($FeCl_3.6H_2O$, 5% W/V in 0.1 N HCl) were added to the tubes, followed by vortexing. The color formation was recorded either using a qualitative scale (visualization) or spectrophotometrically at 435 nm. For spectrophotometric determinations, the stopped reaction mixture was centrifuged (10,000×g, 4°C, 15 min) and the supernatant was measured using a spectrophotometer (Optizen POP, Mecasys Company, Korea). Test tubes containing all the reagents without cells were used as blank. All test tubes were prepared in triplicate.

Genus specific identification of bifidobacteria isolates

The genus specific identification of the purified isolates was performed according to the protocol described by Kharchenko *et al*. (2015). The conventional PCR targeting the gene *xfp* was applied. After overnight reactivation at 37°C anaerobically, each presumptive pure bifidobacteria isolate was washed twice with PBS (pH 7.2) at 6,000×g at 4°C. According to the manufacturer guidelines, the genomic DNA from each washed isolate was extracted using E.Z.N.A. bacterial DNA Kit (Omega Bio-Tek, USA). The extracted DNA was then amplified by PCR (Eppendorf, Germany) using the primers *xfp*-F (5′-CGGCTGGCAGTCCAACAA-3′) and *xfp*-R (5′- GGTTGTTCTTGATGATGTCGG-3′) with an expected PCR product size of around 704 bp (Kharchenko *et al*., 2015). The PCR consisted of 35 cycles of 95°C for 30s, 57°C for 30s, and 72°C for 40s, with an initial denaturation (95°C for 5 min) and a final extension (72°C for 4 min). Both positive and negative controls were included in PCR to validate the results. The PCR amplified products were separated by electrophoresis (Cleaver Scientific Ltd., UK) on 1% agarose gel and visualized using a gel documentation system (Cleaver Scientific Ltd., UK) after staining with ethidium bromide.

Biochemical tests for identification of the isolates

The biochemical tests for identification of all the isolates were carried out using a bifidobacteria specific biochemical identification kit (HiMedia Laboratories Pvt. Ltd., India). The target isolates were grown overnight in MRS broth containing 0.05% L-cysteine. HCl anaerobically at 37°C and the cultured broth was diluted with fresh MRS broth to achieve an optical density of 0.1 at 620 nm. Each well of a strip was filled up with 50µl of diluted cultured broth of the respective isolate and incubated anaerobically at 37°C for 24h. The obtained result was entered into the supplied result entry datasheet and the identification was confirmed by the interpretation chart and identification index.

Measurement of growth dynamics

The growth curve of the target bifidobacteria isolates was observed by monitoring absorbance and pH of the cultured media at different interval of incubation. After reactivating the frozen isolates for at least two times, the overnight culture of the respective isolates were inoculated at 1% (v/v) in a test tube containing 10 ml of MRS broth supplemented with 0.05% L-cysteine. HCl and incubated at 37°C for 24h under anaerobic condition. Test tubes without inoculum were used as control. At every 6 h interval, absorbance and pH of the cultured media was measured at 660 nm using a spectrophotometer and pH meter (Hanna Instruments, Inc. USA), respectively. The experiment was carried out in triplicate. The exponential/logarithmic phase growth rate of each isolate was calculated using the following formula as described by Kask *et al*. (2003):

$$\mu = \frac{\ln X - \ln X0}{\Delta t}$$

Where, $\ln X$ and $\ln X_0$ indicates the absorbance value of the cultured media in the beginning and end of the exponential phase, respectively; Δt indicates time difference between two observations; µ is the specific growth rate.

Statistical analysis

The data generated from the study were inserted into excel sheet (Excel 2007, Microsoft, Redmond, Washington, USA) and were analyzed with the add-in software yStat 2008 (Shinya Yamazaki, Koriyama, Japan) as per the requirement of the study. One-way analysis of variance (ANOVA) with SNK multiple range tests among means was carried out. All tests of significance were two-tailed, and p<0.05 were considered as significant.

Results and Discussion

Isolation of presumptive bifidobacteria based on cultural and morphological characteristics

Twenty colonies were randomly selected and purified by subsequent anaerobic subcultures on bifidobacteria selective media containing mupirocin, a highly bifidobacteria resistant antibiotic. The isolates were coded as BBC-1 to 20, where BBC stands for Bifidobacteria from Buffalo Milk Curd. The selected colonies were white and glossy, convex, and round or irregular. All the isolates were found as non-motile, catalase negative, gram positive and rod- or v-shaped. These cultural and morphological characteristics indicated the isolates as bifidobacteria which is supported by several reviews (Biavati *et al*., 2000; Leahy *et al*., 2005) and research article (Young *et al*., 2011).

Mupirocin resistance ability of presumptive bifidobacteria isolates

All the presumptive bifidobacteria isolates showed resistance against the antibiotic mupirocin. The inhibitory zones produced by the isolates were ranged from 3 to 7 mm. Since the zone diameter for each isolate was less than 10 mm, they

were considered as mupirocin resistant. The antibiotic mupirocin is active against certain Gram-negative and Gram-positive bacteria, including microorganisms that are used in fermented dairy products and functional foods, such as *Streptococcus* sp., *Lactococcus* sp., and *Lactobacillus* sp. and, therefore, widely used in selective media for the isolation of bifidobacteria (Simpson *et al*., 2004). The bifidobacteria exhibit a phenotype of generally high resistance to this antibiotic. The major amino acid residues of the isoleucyl-tRNA synthetase gene (IleS) encoded protein are apparently crucial for bifidobacteria to exhibit resistance against mupirocin (Serafini *et al*., 2011).

Phosphoketolase activity of the isolates

The phosphoketolase activity of all the 20 isolates has been displayed in Fig. 1. The isolates having strong phosphoketolase activity developed reddish-violet color. Conversely, weak or no activity showed dark yellow or light yellow color (analogous to the blank), respectively (Fig. 1a). The visualization results were reflected in the absorbance value of the corresponding isolates as illustrated in Fig. 1b. The intensity of color formation among the isolates varied significantly (p<0.05). The intensity was very high in seven isolates (BBC-1, 2, 5, 10, 12, 13 and 16) and the recorded absorbance value ranged from 1.63 to 1.89. The moderate intensity (absorbance >0.5 but <1.0) was observed in four isolates (BBC-3, 17, 18 and 19). The remaining nine isolates showed weak intensity (absorbance ranged from 0.16 to 0.38). Several studies have confirmed the phosphoketolase test as a reliable test for the genus identification of bifidobacteria (Vlkova *et al*., 2002). Bifidobacteria can be distinguished from other bacterial groups like lactobacilli, actinomycetes, and anaerobic corynebacteria by a particular metabolic pathway, the bifid shunt (Scardovi and Trovatelli, 1965), where the key enzyme is fructose-6-phosphate phosphoketolase. The demonstration of phosphoketolase activity serves as a taxonomic tool in the identification of the genus. This key enzyme in the glycolytic fermentation cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate. Some bifidobacteria strains have reported showing weak color intensity (absorbance value around 0.5) during phosphoketolase assay (Orban and Patterson, 2000; Vlkova *et al*., 2002). Therefore, the moderate color intensity showed by the four isolates in the present study is in agreement with these findings.

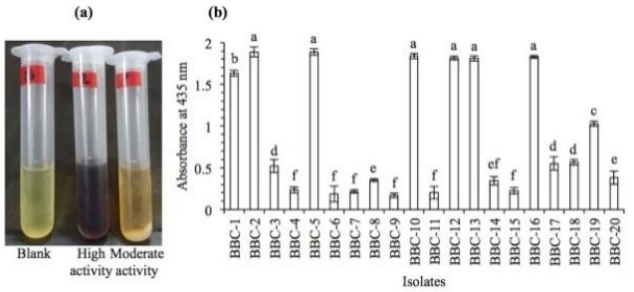

Figure 1: Phosphoketolase activity of presumptive bifidobacteria isolates. Representative image of color developed (blank, high and moderate) by bidobacteria isolates during phosphoketolase assay (a); Color intensity of all bifidobacteria isolates expressed as absorbance at 435 nm (b); The values are the mean±SD; bar with different small letter (a, b, c, d, e or f) differs significantly (p<0.05).

Genus specific identification of bifidobacteria isolates

The genus specific identification of all the isolates was performed by amplifying the target *xfp* gene. As shown in Fig. 2, out of 20 isolates fourteen (BBC-1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 15, 16, 17, 18) were amplified well with the phosphoketolase activity encoded *xfp* gene. A clear band of around 704 bp was appeared in each positive isolate. The phosphoketolase activity encoded gene *xfp* has widely been using for identifying bifidobacteria as genus level (Yin *et al*., 2005). Several primers have been designed to amplify the gene of which *xfp*-F and *xfp*-R have recently been reported by Kharchenko *et al*. (2015). The present results are in agreement with these studies. However, it is noteworthy that genus specific primer may not be reliable or sufficient to identify bifidobacteria (Kharchenko *et al*., 2015). Therefore, partial 16S rRNA gene sequencing is essential.

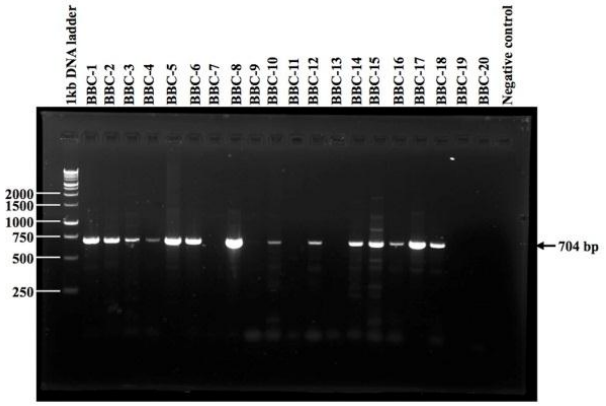

Figure 2: Genus specific identification of presumptive bifidobacteria isolates. Conventional PCR Amplicons targeting *xfp* gene were produced by conventional PCR and separated on 1% agarose gel resulted around 704 bp PCR product (indicated by arrow).

Comparison among mupirocin resistance ability, phosphoketolase activity and genus specific identification of bifidobacteria isolates

The mupirocin resistance ability, phosphoketolase activity and presence of *xfp* gene in all the 20 isolates have been summarized in Table 1. Although all the isolates showed resistance to mupirocin, their phosphoketolase activity and *xfp* gene identification profile did not correspond to each other. Therefore, in the present study the isolates demonstrated positive response against all the parameters tested were only considered as *Bifidobacterium* sp. Of the 20 isolates, six were found positive for both *xfp* gene and phosphoketolase activity. In contrary, four isolates yielded negative results to both the *xfp* gene and phosphoketolase activity. However, five isolates were identified positive for *xfp* gene but negative for phosphoketolase activity, and two isolates showed negative response to *xfp* gene but positive and/or moderate phosphoketolase activity. Although the remaining three isolates (BBC-3, 17 and 18) showed positive response to *xfp* gene, they were also excluded as *Bifidobacterium* sp. due to moderate phosphoketolase activity. Thus, the six isolates (BBC-1, 2, 5, 10, 12 and 16) exhibited positive response to mupirocin resistance, phosphoketolase activity and genus specific identification test were further selected for biochemical identification and growth dynamics study.

Table 1. Summary of mupirocin resistance, phosphoketolase activity and genus specific identification test

Isolates	Mupirocin resistant	Phosphoketolase activity	*xfp* gene identification
BBC-1	+	+	+
BBC-2	+	+	+
BBC-3	+	+/-	+
BBC-4	+	-	+
BBC-5	+	+	+
BBC-6	+	-	+
BBC-7	+	-	-
BBC-8	+	-	+
BBC-9	+	-	-
BBC-10	+	+	+
BBC-11	+	-	-
BBC-12	+	+	+
BBC-13	+	+	-
BBC-14	+	-	+
BBC-15	+	-	+
BBC-16	+	+	+
BBC-17	+	+/-	+
BBC-18	+	+/-	+
BBC-19	+	+/-	-
BBC-20	+	-	-

+, Positive; +/-, Moderately positive; -, Negative

Biochemical identification of bifidobacteria isolates

The biochemical test results of six bifidobacteria isolates have been presented in Table 2. Based on the results, five isolates were identified according to the interpretation chart provided by the biochemical test kit manufacturer. The isolates BBC-1, BBC-12 and BBC-16 were identified as *B. mongoliense*, and BBC-2 and BBC-10 as *B. pseudolongum* subsp. *globosum*. The only isolate BBC-5 was not matched with the interpretation chart. In a recent study, findings revealed that Italian cheeses produced from raw milk derived from cow, buffalo, sheep, and goat are dominated by *B. mongoliense* and *B. crudilactis* and *B. pseudolongum* subsp. glob*osum* (Milani *et al*., 2019). The bifidobacteria isolates identified in this study are also greatly supported by the findings.

Table 2. Biochemical identification of bifidobacteria isolates

Test	Bifidobacteria isolates					
	BBC-1	BBC-2	BBC-5	BBC-10	BBC-12	BBC-16
Catalase	N	N	N	N	N	N
L-Arabinose	P	P	N	P	P	P
Cellobiose	P	N	P	N	N	P
Fructose	N	P	N	P	N	N
lactose	P	P	P	P	P	P
Maltose	P	P	P	P	P	P
Mannose	N	N	N	N	N	N
Mellibiose	P	P	P	P	P	P
Raffinose	P	P	P	P	P	P
Sucrose	P	P	N	P	P	P
Xylose	N	P	N	P	N	N
Salicin	P	N	P	N	P	P

P: Positive; N: Negative; BBC-1, BBC-12 and BBC-16 as *B. mongoliense*; BBC-2 and BBC-10 as *B. pseudolongum* subsp. *globosum*; BBC-5 was not matched with the interpretation chart provided by the manufacturers.

Growth curve and exponential phase growth rate of bifidobacteria isolates

The growth curve and exponential phase growth rate of six bifidobacteria isolates have been illustrated in Fig. 3. The growth curve of all the six bifidobacteria isolates were constructed by monitoring pH and absorbance of the cultured media at different time points of incubation and has presented in Fig. 3 (a-f). After 24 h of incubation, the highest absorbance value (1.89±0.08) was observed in isolate BBC-1 (Fig. 1a) and the lowest value (1.51±0.05) was detected in BBC-10 (Fig. 1d). The pH of the cultured media of all isolates was decreased gradually from the initial 6.38±0.03 to a range of 3.98±0.03 to 4.31±0.04 (Fig. 1a-f). The exponential phase started within first 6 h of incubation and end after 12h. During exponential growth, the logarithm of bacteria population varies linearly. Thus, the exponential phase growth rate was calculated based on the absorbance value measured at different time point of incubation and has been shown in Fig. 1g. The exponential phase growth rate of tested isolates varied significantly (p<0.05). The highest growth rate (0.34±0.02/h) was achieved by the isolate BBC-1 and the lowest rate (0.16±0.01/h) was observed in BBC-2. The growth rate of BBC-1 was found significantly (p<0.05) different from the remaining five bifidobacteria isolates. There was no significant difference between growth rate of BBC-2 and 10, and BBC-5, 12 and 16. Because they might be belong to the same species as identified by biochemical test (Table 2). In the present study, the growth rate of the tested isolates was low. The reason could be the use of glucose present in MRS broth as a sole carbon source. However, the present findings and explanation are supported by Rada *et al*. (2002) who reported specific growth of bifidobacteria ranged from 0.25 to 0.44/h and bifidobacteria strains from dairy origin propagated slowly on glucose.

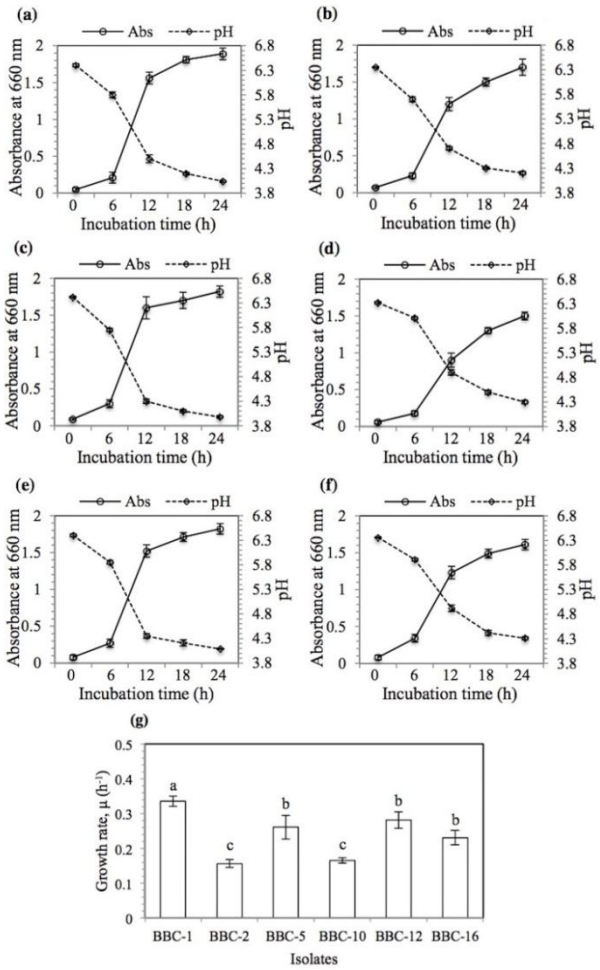

Figure 3: Growth curve (a-f) and exponential phase growth rate (g) of *Bifidobacterium* sp. BBC-1 (a), BBC-2 (b), BBC-5 (c), BBC-10 (d), BBC-12 (e), BBC-16 (f) based on absorbance at 660 nm and pH of the cultured media during incubation. The values are the mean±SD; bar with

different small letter (a, b, c, d, e or f) differs significantly (p<0.05).

Conclusions

In the present study, a naturally fermented traditional buffalo milk curd obtained from *Charfassion* upazila of *Bhola* district, Bangladesh was explored to isolate and identify bifidobacteria, a potent probiotic candidate. A substantial number of isolates were confirmed as *Bifidobacterium* sp. based on their cultural, morphological, biochemical, phosphosketolase and genus specific characteristics, suggesting that buffalo milk curd is as a prospective niche of bifidobacteria. The source of bifidobacteria in the product could be mammalian secretion and/or environment during the entire production process. Since buffalo milk curd has already been using for human consumption, *Bifidobacterium* sp. obtained from this study could be considered as safe. However, identification at species level and evaluation of probiotic potential are crucial for future industrial application of these isolates.

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