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Article

Probiotic properties of *Bifidobacterium* species isolated from mother's milk and infant feces

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Abstract: Probiotics are live microorganisms that provide health benefits when consumed. *Bifidobacteria* are one of the major genera of bacteria that make up the colon flora in mammals and have great beneficial impact on health. This study was performed to culture and characterize some potential *Bifidobacterium* sp. isolated from mother's milk and infant feces. Eleven isolates were presumptively identified as *Bifidobacterium*s sp. based on morphological characteristic and biochemical properties such as catalase, oxidase, Esculin test, Gelatinase activities and production of gas from glucose. Probiotic potentials of the isolates were investigated through probiotic potentiality tests e.g. p^H tolerance test, NaCl tolerance test, bile salt tolerance test, antimicrobial activity, growth rate in gastric juice, antimicrobial susceptibility (Antibiogram). Four of the isolates BI-3, BI-5, BI-9, and BI-14 were observed as potential probiotic based on their probiotic activities. These isolates showed resistance to stomach pH (pH 3.0), tolerance against 0.3% bile concentration and gastric juice tolerance. Most of the isolates were able to grow at 1- 6% NaCl concentration and the growth declined with the increase of the salt concentration. Proteolytic activity was measured to estimate the probable role of probiotics in protein digestion. For this purpose milk protein digestion was tested and these isolates had showed better proteolytic activity than others. Antimicrobial activity against pathogens showed better probiotic properties. These isolates were found to have moderately antibiotic susceptible to common antibiotics. This experiment revealed that these four isolates BI-3, BI-5, BI-9, and BI-14 have good probiotic properties and can be used as probiotics after successful molecular identification and completion of animal trial.

Keywords: *Bifidobacterium* sp.; mother's milk; infant feces; probiotics

1. Introduction

Bifidobacterium is a genus of gram-positive, non-motile, often branched anaerobic bacteria inhabit in vagina and gastrointestinal track (Mayo *et al.*, 2010; Schell *et al.*, 2002). Various species of Bifidobacteria are common members of the human gut micro flora, comprising up to 3% of the total fecal adults microflora. They are more numerous in the infant gut, where they form up to 91% of the total microflora in breast-fed babies and up to 75% in formulated infants (Harmsen *et al.*, 2000). These are used in the preparation of probiotic products (Boudraa *et al*., 1990; Lievin *et al*., 2000; Requena *et al*., 2002).

The term "probiotic" originated from the Greek word "probios" meaning "for life" (as opposed to "antibiotic," which means "against life") (Longdet *et al.*, 2011).The history of probiotics began with the history of man by consuming fermented foods that is well known in Greeks and Romans (Gismondo *et al*., 1999; Guarner *et al*., 2005) . Our normal flora contains more than 400 species of bacteria that endow with many beneficial functions

(Mahan *et al*., 2004). So Probiotics can be simply defined as naturally happening microorganisms consumed as a food component or dietary supplement that provide good health. Probiotics are microbial food supplements, when administered in adequate amounts; confer health benefits to consumers by maintaining or improving their intestinal microbial flora (Salminen *et al*., 1998; Reid *et al*., 2003).

When selecting probiotics, some criteria must have to be met by the probiotics organisms such as resistance to the enzymes in the oral cavity (e.g. lysozyme) and should also have the ability to resist the digestion process in the stomach and intestinal tract and arrive at the site of action in a viable physiological state and adhesion to mucosal surfaces. Cultures must have Generally Regarded as Safe (GRAS) status if they are to be used as probiotics and also meet a number of good technological properties e.g. easy propagation and incorporation into foods and long term survival and safe in food products and clinically validated and documented health effects. Probiotic bacteria are frequently used as the active ingredient in functional foods such as bio-yoghurts, dietary adjuncts and health-related products (Brassart and Schirin, 1997). The health benefits (for consumers) attributed to probiotic bacteria can be categorized as either nutritional benefits or therapeutic benefits. Nutritional benefits include: their role in enhancing the bio-availability of calcium, zinc, iron, manganese, copper and phosphorus, increasing the digestibility of protein and synthesis of vitamins (Sudha Rani and Srividya, 2014). The therapeutic benefits of probiotics reported include: treatments of conditions including gastrointestinal disorders (Biller *et al*., 1995) ; hyper-cholesterolaemia (Noh *et al*., 1997); and lactose intolerance (Mustapha *et al*., 1997); suppression of pro-carcinogenic enzymes (McConnell and Tannock, 199; Fujisawa and Mori, 1997); inhibitory efects on tumour cells (Kechagia *et al*., 2013); immunomodulation (Perdigon *et al*., 1995); and the treatment of food-related allergies (Majamaa and Isolauri, 1997; Kechagia *et al*., 2013).

Studies involving *Bifidobacterium* species identification from human usually require enumeration of these organisms in feces (Silvi *et al*., 1996). Due to this wide probiotic activity, the *Bifidobacterium* have large industrial and medical importance (Richardson, 1996; Takahashi *et al*., 2004) and there has been an increased interest in the use of *Bifidobacteria* as feed supplements (Shah, 2000). Recently, research has focused on identifying new strains of *Bifidobacterium* with health-promoting properties. It is nevertheless, important to confirm the safety of any newly identified probiotic strains (Martin and Cabbage, 1992; Collins and Gibson, 1999).

Being a developing country like Bangladesh, considerable number of people lives below the poverty line and a substantial population remains malnourished. Development of various probiotic products, such as fermented milk drinks, yoghurt, cheese, ice-cream etc. with defined probiotic culture would be able to confer health benefits of mass and common people of Bangladesh. The main objectives of our study is to isolation and characterization of *Bifidobacteria* from infant feces and mother's milk sample through different biochemical tests, and end with analysis of probiotic properties of those isolates.

2. Materials and Methods

2.1. Sampling and Maintenance Subjects

The samples were collected in sterile container and stored on ice until delivery to the laboratory. The procedure for isolation start immediately, once delivered to the laboratory. Pour plate technique was used to isolate the organisms, samples were used directly and also diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water and 1 ml aliquot of the samples and dilutions were plated into Highcrome Bifidobacterium (M1960-500G, HIMEDIA) (pH 6.2 and pH5.5), Modified Bifidobacterium agar (M1858-500G, HIMEDIA) (pH 6.5).

The plates were incubated at 37°C for 2 days under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact), after incubation individual colonies were selected and transferred into sterile broth mediums. The following step is purifying the selected colonies with streak plate technique. The isolates were examined according to their colony morphology, catalase reaction and gram reaction. Gram positive and catalase negative cocci and bacilli colonies were taken to the glycerol stocks as *Bifidobacteria*.

2.2. Bacterial characterization

2.2.1. Morphological characterization

Bacterial plates were purified by using subculturing technique on MRS agar media, and the colony morphologies (color, shape and size) were examined in nude eye, however microscopic observation was needed to separate one colony to another. According to the protocol of Erkus, gram staining was done with some modifications (Erkus, O. 2007).

2.2.2. Biochemical characterization

2.2.2.1. Catalase test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme (Holt *et al.,* 1994). Catalase test was performed to isolates in order to see their catalase reactions.

2.2.2.2. Oxidase test

The enzyme oxidase, present in certain bacteria catalyses the transport of electron from donor bacteria to the redox dye tetra-methyl-p-phenylenediaminedihydrochloride. The dye in the reduced state has a deep purple color. Basically this is a test to see if an organism produces cytochrome C oxidase. The positive result was indicated by the production of dark blue color within 7 seconds (Isenberg HD 2004).

2.2.2.3. Indole test

10 ml tryptophan medium were poured in each McCartney bottle, after the media were autoclaved (for 15 minutes in 15 Ibs pressure at 121°C) inoculation processes were done. Then the inoculated media were left for incubation 24 hours at 37°C. *E coli* ATCC 8739 used as a positive control and a lab isolates of *Pseudomonas aeruginosa* was used as a negative control. The amino acid tryptophan can be broken down by enzyme tryptophanase to form Indole, pyruvic acid and ammonia as end products. Tryptophanase differentiates indole-positive enterics (e.g, *E. coli*) from closely related indole-negative enterics. Indole can be detected with Kovac's reagent (indole reagent) (Bachoon *et al*., 2008).

2.2.2.4. Gelatin liquefaction test

Gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy gelatin (Leboffe and Pierce, 2010). Gelatin is a protein derived from the connective tissues of vertebrates, that is, collagen, it is produced when collagen is boiled in water (Harley *et al.,* 2005), and gelatin hydrolysis indicates the presence of gelatinase.

2.2.2.5. Esculin test

Bile Esculin is a selective and differential medium; it contains bile (which inhibits Gram-positive staphylococci) and sodium azide (which inhibits Gram-negative bacteria), also it contains esculin and peptone as nutrient sources and ferric citrate as a color indicator (Grabsch *et al.,* 2008). Esculin is a carbohydrate linked to an alcohol. Many organisms can hydrolyze esculin but esculet in as a by-product. Esculin in reacts with ferric citrate to form a dark (black) coloration.

2.2.2.6. Gas production from glucose

In order to determine the homofermentative and heterofermentative characterization of isolates, CO_2 production from glucose test was applied (Choudhury, 1999). Citrate lacking TPY broths and inverted Durham tubes were prepared and inoculated with fresh cultures. Then the test tubes were incubated at 37°C for 5 days, gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO_2 production from glucose.

2.2.2.7. Growth at different temperatures

TPY containing bromecresol purple indicator, was prepared and transferred into tubes as 5 ml. Then 50µl of overnight cultures inoculated and incubated for 7 days at 10°C, 15°C, and 45°C. During these incubation time cells growth at any temperatures was observed by the change of the color of media from purple to yellow.

2.2.2.8. Carbohydrate fermentations

Sugar fermentation tests were done according to modified protocol of Erkus (Erkus, 2007). At first every sugar was dissolved in deionized water at a final concentration of 5% (w/v), then sterilization of sugar solutions were done by filter paper with 0.22µm pore diameter. TPY broth (p^H 6.5) was taken into Mac Carty tube and carbohydrate fermentation broth were placed and the medium was autoclaved at 121°C for 15 min. 1 ml different sugar solutions were inoculated into different tubes and 200µl overnight liquid cultures were inoculated into the broth medium. Incubation was performed anaerobically at 37°C for 24 h.

2.3. Probiotic properties of isolates

For the determination of probiotic properties of isolates the major selection criteria were: resistance to low p^H, tolerance against bile salt and the antimicrobial activity.

2.3.1. Resistance to low pH

Resistance to p^H 3 is often used in vitro assays to determine the resistance to stomach p^H, because the foods remain in stomach for 3 hrs, this time limit was taken into account (Prasad *et al*., 1998). For the determination of growth in different p^H 100µl overnight cultures of the isolates were inoculated into TPY broth with varying p^H ranging from 2.5, 3.0, 4.0, 5.0, 6.0, and 7.0. The low p^H value of the TPY medium was adjusted by 1M HCl. The inoculated broths were then incubated in anaerobic condition for 24 h at 37°C. Growth of the probiotic bacteria was visible by comparing with that of TPY broth (Graciela *et al.,* 2001). Viable microorganisms were enumerated at the 0, 1, 2 and 3 hours with pour plate techniques.

2.3.2. Growth at different NaCl concentrations

Isolates were tested for their tolerance against different NaCl concentrations from 4% to 6.5% NaCl to determine the NaCl tolerance. After sterilization, each test tube was inoculated with fresh overnight culture of *Bifidibacterium* spp, incubated anaerobically and after 20-24 h the probiotic bacteria was compared with TPY broth (Graciela *et al.,* 2001).

2.3.3. Bile tolerance activity

Intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h (Prasad *et al*., 1998). In the experiment TPY medium containing 0.3% bile (Oxoid) was inoculated with active cultures (incubated for 16-18 h). During the incubation for 4 h, viable colonies were enumerated for every hour with pour plate technique and also growth was monitored at OD_{620}(Thermo Multiskan EX).

Growth rate of bacterial culture was determined in TPY broth containing different levels of bile salt concentration (0.3%, 0.5%, 1%, 1.5%, 2% and 2.5%). Inoculum of probiotic bacteria was prepared in TPY broth by overnight incubation and 1% (v/v) fresh culture was added to each tube containing bile salt and then incubated anaerobically overnight at 37°C. After 24 h growth of probiotic bacteria was compared with TPY broth (Gilliland *et al.,* 1984; Chateau *et al.,* 1994) and the survival rates of the isolates were measured by spectrophotometer at OD_{620}.

2.3.4. Assay for determination of gastric juice tolerance

NaCl (2 g), pepsin (3.2 g) were adjusted at a final p^H with HCl without dilution and taken to 1L with distilled water. As a control, artificial gastric juice was adjusted at a final p^H 6.6 with 5N NaOH. Sterilization was done by filtration. The *Bifidibacterium* spp. was grown in TPY broth at 37°C for 16 h. Then the artificial gastric juice having p^H 2.22 and p^H 6.6 were inoculated with the 2% (v/v) bacterial suspension. Both the media were incubated at 37°C and samples were taken at 0, 1, 2, 3, and 4h and after 24 h for cell viability was measured at 620 nm.

2.3.5. Antibiotic susceptibility test

Susceptibility testing was based on the agar overlay disc diffusion test described by Charteris *et al*., 1998 as modified by Aymerich *et al*., 2006. Briefly, *Bifidibacterium* spp. were grown overnight in TPY broth at 37ºC under anaerobic conditions. Petri dishes containing 20 mL of MHA allowed to solidify at room temperature and swabbed with inoculated MRS. Antibiotic discs were placed onto the overlaid plates and all plates were incubated at 37^0C for 24 h under anaerobic condition. All isolates were screened for their susceptibility to penicillin G (10 µg), ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg) and chloramphenicol (30 µg) Nitrofurantoin (300 µg) Inhibition zones diameters of antibiotics were compared to those defined by Vlkova *et al.,* 2006.

2.3.6. Antimicrobial activity of bacteriocin produced by *Bifidobacterium* spp.

2.3.6.1. Agar well diffusion method

Agar well diffusion method was modified (Schillinger and Lucke, 1989; Toba *et al.,* 1991) and used to detect antimicrobial activities of bacteriocin produced from *Bifidobacterium* spp. These assays were performed in duplicate. The plates were poured with 20 mL nutrient agar media (NA). Eight different human pathogens belonging to both gram-positive and gram negative groups such as *Bacillus cereus* ATCC 10876,

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 8739, *Salmonella typhi* ATCC 65154, *Shigella flexneri* ATCC 12022, *Enterobacter faecalis* ATCC 29212, *Listeria monocytogenes* were grown in nutrient broth (NB) for 24 hours and spread on the surface of nutrient agar plate. Four wells (7 mm in diameter) of each plate were made by using a sterile borer and 10 µl of the supernatant of the *Bifidibacterium* spp. were placed into four well.

2.3.7. Proteolytic activity

Proteolytic activity of bacterial culture was determined in 1%, 4% PCA and 1%, 4% YMA. Plates were incubated anaerobically at 37°C after 24 h growth of the proteolytic bacteria was visible by zone formation (Chateau *et al.*, 1994).

3. Results

3.1. Samples

A total of twenty five mothers and their breastfeeding infant were included in this study. They were selected according to the following criteria: (i) healthy women without present or past critical health conditions; (ii) normal, full-term pregnancy; and (iii) absence of infant and/or maternal prenatal problems, including mastitis. Feces sample was taken from infants within the age range from two days to two months (Table 1). None of the subjects enrolled in this study had received an antibiotic treatment during pregnancy or after birth.

3.2. Identification of the bacterial isolates

The selected isolates were observed under optical microscopy to determine their morphology and gram staining results and were presumptively identified as *Bifidobacterium* spp. using morphological and biochemical characteristics.

3.2.1. Colony characteristics and morphological features

Different isolates showed different morphological characters. There were different in shape and color of colony (Table 2).

3.2.2. Biochemical characterization

In HighCrome Bifidobacterialmedum (M1960-500G, HIMEDIA) *Bifidobacteria* show Greenish, Blueish or whitish slimy colony and under microscopic examination *Bifidobacteria* appears as small rod or small Y shape bacteria. According to these characteristics some isolates BI-3 to BI-17 selected for their biochemical characteristics. Additionally, they were tested for Catalase, Oxidase, Esculin test, Gelatinase activities, production of indol, and production of gas from glucose (Table 3).

Morphological identification and through Biochemical characterization 11 isolates were selected for carbohydrate fermentation and other tests.

3.2.2.1. Carbohydrate fermentation test

The carbohydrates fermentation was determined on carbohydrate broth supplemented with 1% of the following carbohydrates: Lactose, Sucrose, Xylose, Arabinose, Sorbitol, Glucose, Galactose, Mannose, Cellulose, Ribose, Selicin, Mannitol, and Trehalose (Table 4).

3.2.2.2. Growth at different temperatures

50µl of cultures inoculated and incubated for 7 days at 10°C, 15°C, 37°C, 42°C and 45°C. All isolates were grown at 37°C and 42°C (Table 5).

3.3. Probiotic activity of *Bifidobacterium* spp.

3.3.1. p^H tolerance test

P^H tolerance of each bacterial isolates was measured by growing each isolates in a varying p^H environment. It was found that isolates BI-3, BI-5, BI-9, BI- 14, could grow up to pH 3.0. Even isolates BI-3 and BI-14 can also showed growth at pH 2.5. That indicates their acid resistance capacity (Table 6).

3.3.2. Measurement of bile salt tolerance

Bile salt tolerance of each bacterial isolates was measured by growing each isolates in a varying 0.3% 0.5% 1.0% 1.5% 2.0% 2.5% bile salt concentration .It was found that most of the isolates are resistant to a bile salt concentration of 0.3% - 2.5% .That indicates their bile salt resistance capacity (Table 7).

3.3.3. Measurement of salt (NaCl) tolerance

Tolerance to sodium chloride was determined by testing their ability to grow in the presence of different concentrations of NaCl [1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0%, 8.0% and 9.0% (w⁄v)] as previously described (Collado *et al*., 2005). The assayed concentrations of NaCl included those present in physiological conditions (0.5%) and food fermentations (up to 6–8%). It was found that all isolates have a salt tolerance of 1% NaCl. According to the tests result four isolates BI-3, BI-5, BI-9 and BI-14 selected for further antimicrobial and antibiotic susceptibly tests. These four isolates were much more resistance to acid also showed comparatively high tolerance to NaCl Gastric juice tolerance and Bile salt tolerance (Table 8).

3.3.4. Antimicrobial activity measurement

The selected isolates were examined for their antimicrobial commotion against the indicator microorganisms .Eight different human pathogens belonging to both gram-positive and gram negative groups such as *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Salmonella typhi* ATCC 65154, *Shigella flexneri* (2b) ATCC 12022, *Enterobacter faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 19112, *S. enteritidis* ATCC 13076, *B. bifidum* ATCC 15697 were used in this study as test pathogen. The diameter of inhibition zones confirmed that all the isolates have antibacterial consequence against the indicator microorganisms (Figure 1).

3.3.5. Gastric juice tolerance

The viability of isolates to gastric juices is shown in Figure 2 and 3 All isolates BI-3, 5, 9 and 14 had better viability in gastric juice for 3 h. Isolates BI-5,14 had the highest survival rates in gastric juice. Furthermore, Figure 4 showed that all isolate BI- 3, 5, 9, and 14 was capable to survive in gastric juice at pH 2.2 for 24 hours. After 8 hours all the isolates declined in number and isolate BI-3 showed highest survival rate.

3.3.6. Antibiotic susceptibility

The antimicrobial susceptibility of intestinal microorganisms is an important criterion for selecting an organism as a probiotic. Compared with lactobacilli, strains usually used as probiotics, *Bifidobacteria* appear to be more susceptible to antibiotics. Antibiotic resistance test of different isolates showed different result over different antibiotics (Figure 4).

3.3.7. Measurement of proteolytic activity

Proteolytic activity was measured to estimate the probable role of probiotics in protein digestion. For measuring proteolytic activity digestion with milk protein was tested. It was found that organism BI- 3, 5, 9 and 14 has higher proteolytic activities. Isolate BI-3 and BI-5 showed proteolytic activity at 1% PCA that was 12 mm in diameter and showed highest proteolytic activity in 1%YMA 25 and 23 mm in diameter. In 1% PCA BI-9 and 14 showed 15 mm clear zone (Table 9).

Table 1. Fecal samples collected from infants.

Sample ID	Infant's Sex	Age
BI-1	M	3 days
BI-2	M	1 month
BI-3	F	2 days
BI-4	F	7days
BI-5	F	3days
BI-6	M	12 days
BI-7	F	8days
BI-8	M	1month
BI-9	M	20days
BI-10	F	3days
BI-11	F	5 days
BI-12	F	20 days
BI-13	F	10days
BI-14	M	5days
BI-15	M	4days
BI-16	M	1 month
BI-17	F	20 days

Sample ID	Infant's Sex	Age
BI-18	F	1 month
BI-19	F	2 month
BI-20	F	15 days
BI-21	F	15 days
BI-22	F	6 days
BI-23	M	17 days
BI-24	M	19 days
BI-25	F	1 month

Table 2. Colony characteristics and morphological features of the isolates.

ID	Colony, Cell morphology
BI-1	Circular pinkish colony,Rods.
BI-2	Circular White colony with zone, Cocci.
BI-3	Bluish green color colony,Rods.
BI-4	Circular White colony with zone, Rods.
BI-5	Circular Bluish colony glazy appearance, Rods.
BI-6	Small rod, bluish green colony.
BI-7	Greenish colony,small rod shape.
BI-8	Greenish colony,small rod shape.
BI-9	Circular Whitish colony,Rods
BI-10	Bluish green color with white center
BI-11	Circular White colony,creamy appearance.
BI-12	Greenish colony,small rod shape.
BI-13	Small rod, Bluish color, glazy appearance.
BI-14	Greenish colony,small rod shape.
BI-15	Circular white creamy colony, Rod shape.
BI-16	Greenish colony,small rod shape.
BI-17	Bluish green color with white center,Rods.
BI-18	White colony,circular,glazy appearance.
BI-19	Bluish color, glazy appearance.
BI-20	Yellowish colony, white center.
BI-21	Circular,pinkish colony.
BI-22	Greenish colony, Glazy appearance.
BI-23	Circular pinkish colony.
BI-24	White colony, circular, glazy appearance.

Table 3. Biochemical characteristics observed of selected bacterial isolates.

	Sample ID																								
Characteristics	**BI-1**	**BI-2**	**BI-3**	**BI-4**	**BI-5**	**BI-6**	**BI-7**	**BI-8**	**BI-9**	**BI-10**	**BI-11**	**BI-12**	**BI-13**	**BI-14**	**BI-15**	**BI-16**	**BI-17**	**BI-18**	**BI-19**	**BI-20**	**BI-21**	**BI-22**	**BI-23**	**BI-24**	**BI-25**
1. Spore formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3. Catalase activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. Oxidase activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5. Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6. Esculin test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7. Gas production from Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Readings were done under anaerobic conditions after 24 h at 37 °C; Key: + = Positive reaction, - = Negative reaction.

Table 4. Carbohydrate fermentation test.

Sample ID	Ara	Gal	Xyl	Rib	Cel	Lac	Man	Sel	Tre	Glu
BI-3	-	+	-	+	+	+	-	+	-	+
BI-5	-	+	-	+	+	+	+	+	-	+
BI-9	-	+	-	+	+	+	+	+	-	+
BI-10	-	+	-	+	+	-	+	+	-	+
BI-11	-	+	-	+	+	+	-	+	-	+
BI-12	-	+	-	+	+	+	-	-	-	+
BI-13	-	+	-	+	+	+	-	+	-	+
BI-14	-	+	-	+	+	+	+	-	-	+
BI-15	-	+	-	+	+	+	+	+	-	+
BI-16	-	+	-	+	+	+	-	-	-	+
BI-17	-	+	-	+	+	+	-	-	-	+

Readings were done under anaerobic conditions after 24 h at 37 °C; Key: + = Positive reaction, - = Negative reaction.Ara=Arabinose, Gal=Galactose, Xyl=Xylose, Rib=Ribose, Cel=Celbiose, Lac=Lactose, Man=Mannose, Sal=Salicin, Tre=Trehalose, Glu=Glucose.

Table 5. Growth in different temperature.

	Sample ID																								
Growth in different temperature:	**BI-1**	**BI-2**	**BI-3**	**BI-4**	**BI-5**	**BI-6**	**BI-7**	**BI-8**	**BI-9**	**BI-10**	**BI-11**	**BI-12**	**BI-13**	**BI-14**	**BI-15**	**BI-16**	**BI-17**	**BI-18**	**BI-19**	**BI-20**	**BI-21**	**BI-22**	**BI-23**	**BI-24**	**BI-25**
0°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
47°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Readings were done under anaerobic conditions for 7 days at different temperature; Key: + = Positive reaction, - = Negative reaction.

Table 6. p^H tolerance of different isolates while growing on TPY broth.

Sample ID	pH									
	2.5	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**	**6.5**	**7**
BI-3	-	+	+	+	+	+	+	+	+	+
BI-5	-	+	+	+	+	+	+	+	+	+
BI-9	-	+	+	+	+	+	+	+	+	+
BI-10	-	-	+	+	+	+	+	+	+	+
BI-11	-	-	+	+	+	+	+	+	+	+
BI-12	-	-	-	+	+	+	+	+	+	+
BI-13	-	-	+	+	+	+	+	+	+	+
BI-14	-	+	+	+	+	+	+	+	+	+
BI-16	-	-	+	+	+	+	+	+	+	+
BI-17	-	-	-	+	+	+	+	+	+	+

Readings were done under anaerobic conditions after 24 h at 37 °C; Key: + = Positive reaction, - = Negative reaction.

Table 7. Bile salt tolerance of different isolates in TPY broth.

Sample ID	Bile concentration					
	0.3%	0.5%	1%	1.5%	2%	2.5%
BI-3	+	+	+	+	+	+
BI-5	+	+	+	+	+	+
BI-9	+	+	+	+	+	+
BI-10	+	+	+	+	+	+
BI-11	+	+	+	+	+	+
BI-12	+	+	+	+	+	+
BI-13	+	+	+	+	+	+
BI-14	+	+	+	+	+	+
BI-16	-	+	+	+	+	+
BI-17	-	+	+	+	+	+

Readings were done under anaerobic conditions after 24 h at 37 °C; Key: + = Positive reaction, - = Negative reaction.

Table 8. Salt tolerance of different isolates in TPY broth.

Sample ID	NaCl (%)								
	1	2	3	4	5	6	7	8	9
BI-3	+	+	+	+	+	+	+	+	+
BI-5	+	+	+	+	+	+	+	+	+
BI-9	+	+	+	+	+	+	+	-	-
BI-10	+	+	+	+	+	+	+	-	-
BI-11	+	+	+	+	+	+	+	-	-
BI-12	+	+	+	+	+	-	-	-	-
BI-13	+	+	+	+	+	-	-	-	-
BI-14	+	+	+	+	+	+	+	+	-
BI-16	+	+	+	+	+	-	-	-	-
BI-17	+	+	+	+	-	-	-	-	-

Readings were done under anaerobic conditions after 24 h at 37 °C; Key: + = Positive reaction, - = Negative reaction

Table 9. Proteolytic activity of different sample*.

Sample ID		BI-3	BI-5	BI-9	BI-14
Media	4% PCA	20	21	-	-
	1%PCA	20	21	23	25
	1%YMA	25	23	-	-

*****All measured in mm. PCA=Plate Count Agar. YMA=Yeast Milk Agar.

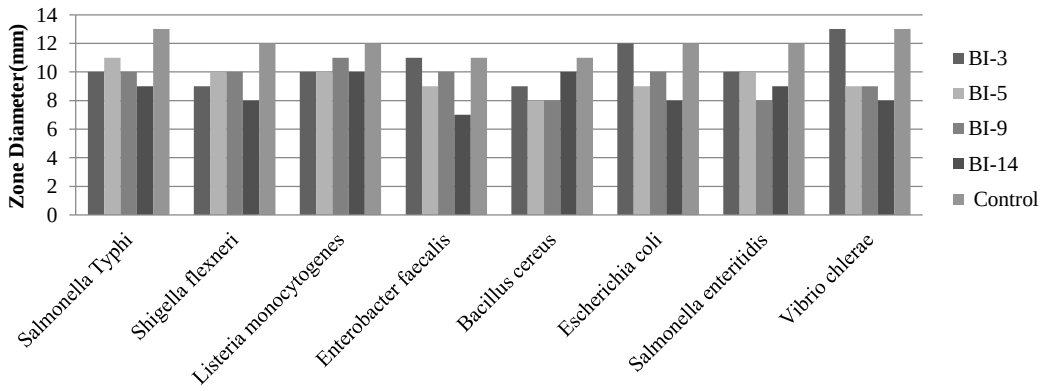

Figure 1. Antimicrobial activity of different isolates after 18 hours culture.

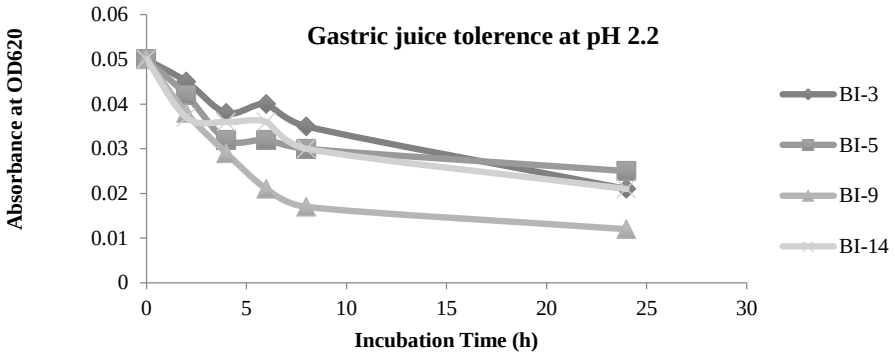

Figure 2. Gastric juice tolerances of the isolates at pH 2.2 through spectrophotometer.

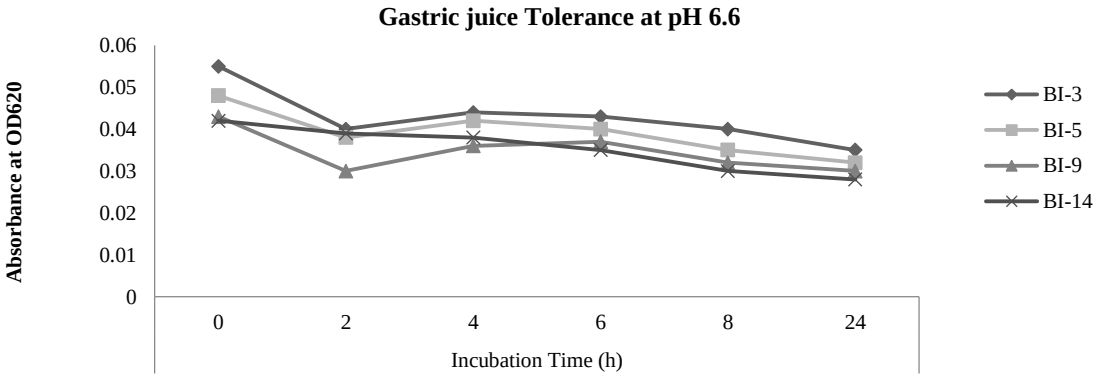

Figure 3. Gastric juice tolerances of the isolates at pH 6.6 through spectrophotometer.

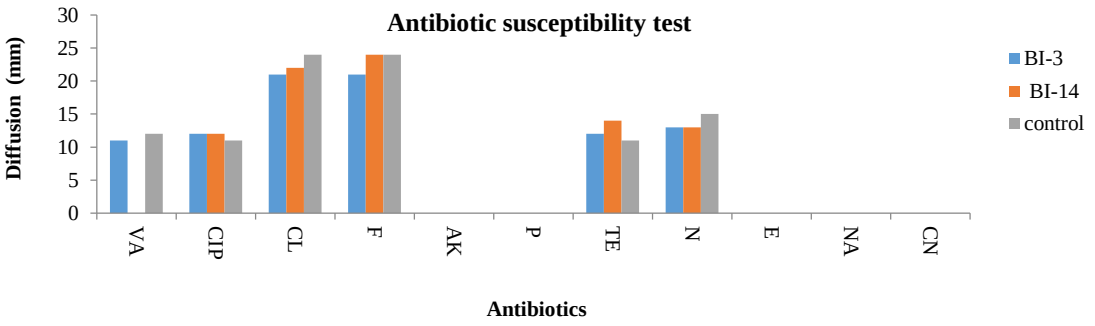

Figure 4. Antibiotic resistance test of different test pathogen.

4. Discussion

The study was designed for characterization and determination of probiotic properties of some *Bifidobacterium* spp. isolated from infant feces and mother's milk. The *Bifidobacterium* spp. are dominance species in the fecal microflora of breastfed infants exclusively due to the bifidigenes factors released from the mother's milk (Modler *et al*., 1990a).The TPY medium was a selective medium for the isolation of *Bifidobacterium* species (Thitaram *et al*., 2005). The stages of the pre-identification based on morphological aspects show that the *Bifidobacteria* develop on TPY medium small colonies without catalase and oxidase activities, indicating that the strains are strictly anaerobic. The cells are Gram positive and bifideform (Scardovi, 1986; Beerens, 1990). The *Bifidobacteria* pleomorphism observed is often associated with the composition of the medium culture (Roy, 2001; Gavini *et al*., 1990) reported that any strain belong's to the *Bifidobacteria* must be nitrate reductase negative, does not form indole, and does not liquify the gelatine. The eleven selected isolates fulfilled the previous characteristic and presumptively identified as *Bifidobacteria* from infant feces and mothers' milk samples .These isolates were selected for the test of other activities. The isolated probiotic bacteria were capable

to grow optimally at 37°C under anaerobic condition but were unable to grow at 5°C, 15°C, 25°C , and 47°C (Pundir *et al*., 2013). Carbohydrate metabolic abilities may vary considerably between bifidobacterial strains (Samelis *et al.,* 2003). However, many of the characterized strains can utilize ribose, galactose, fructose, glucose, sucrose, maltose, and glucose, but generally cannot ferment L-arabinose, rhamnose, sorbitol, trehalose, glycerol, and xylose (Table 4). The criteria for the in vitro selection of potential probiotic were the survival capacity and growth rate of *Bifidobacteria* at different physiological conditions. The tests that were performed were $-p^H$ tolerance test, NaCl susceptibility test, bile salt tolerance test, Anti-microbial susceptibility test, growth rate in gastric juice and antimicrobial activity test (Antibiogram). Although the degree of tolerance required for maximum growth in the GIT is not known, it seems reasonable that the most bile and acid-resistant species should be selected.

The optimal pH of growth of the diverse strains of *Bifidobacteria* was determined by several authors (Beerens, 1990). Infact, germs acidophiles develop better at pH 5 and 6. The results obtained show that the optimum of growth of our strains is p^H 6.5. The growth of isolates were inhibited on TPY medium in p^H 3 but two BI-3 and BI-14 isolates were survive up to p^H 2.5 for 4 hrs. Isolates BI-3, BI-5, BI-9 and BI-14 were capable to survive in low p^H . However the basic p^H (7) decrease the level of the growth rate. These observations indicate that the *Bifidobacteria* prefer the neutral or slightly acid medium (Biavati *et al*., 1992). The temperature of incubation was a significant parameter which defines the bifidobacteria growth. The *Bifidobacteria* of human origin are mesophiles with optimal growth at 36 to 40°C and the temperature also affects the composition of the cell wall fatty acids (Biavati *et al*., 1992).

All probiotic isolates were capable to grow at 1-6% NaCl concentration but failed at 7-9% NaCl concentration. The bile concentration of the human gastrointestinal territory varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h (Prasad *et al*., 1998). According to our findings, every probiotic isolates were able to grow up in 0.15 to 0.3% bile salt concentrations. Resistance to bile salt toxicity is one of the criteria used to select probiotic strains that would be potentially capable of performing effectively in human gastrointestinal tract (Begley *et al*., 2005).

The isolates also showed inhibitory activity against 8 test pathogens (*Salmonella typhi, Shigella flexner, Listeria monocytogenes, Enterobacter faecalis, Bacillus cereus, Escherichia coli, Salmonella,* and *S. enteritidis*) by producing zones of inhibition ranged from 8 mm to 13 mm diameter. That means isolated probiotic strains can produce antimicrobial product which can restrain the growth of pathogenic bacteria. The antimicrobial effect of probiotics could be due to the production of acetic and lactic acids that lowered the overall p^H (Mobarez *et al*., 2008; Bezkorovainy, 2001).

Probiotic bacteria may also have competed for nutrients concurrently by producing hydrogen peroxide and bacteriocins that act as antibacterial agents (Marteau *et al*., 1990). Isolates showed different antibiotic resistance against antibiotic Nitroflurantoin (F300) and Gentamicin (CN10).

Proteolytic activity was found to be highest of zone size 25 mm. Proteolytic activities was measured to estimate the probable role of probiotics in protein digestion. For this purpose milk protein was tested and organism BI-3, BI-5, BI-9, and BI-14 has showed higher proteolytic activity than other isolates.

Comparing to other isolates, isolate no. 3, 5, 9 and 14 have better probiotics properties in terms of p^H tolerance, NaCl tolerance, bile salt tolerance, antimicrobial activity, antibiotic resistance etc. Using these isolates as probiotics strains could have a better health benefits upon consumption. From all the conducted experiments it was observed that, isolated lactic acid bacteria could be used as an excellent candidate for probiotics and finally for probiotic product development.

5. Conclusions

The use of probiotics as food or dietary supplement to help recovery of gastrointestinal disorders is increasing with the discovery of the beneficial effects of these agents. Recently it has been discovered that probiotics have effect on reducing the colon cancer but the reoccurrence of cancer due to such treatments has not yet been established. The commercial use of probiotics, however, has proceeded because essentially no risk is associated with the consumption of well-defined probiotics in foods and many benefits are possible. Perhaps the most compelling evidence for probiotic efficacy is in the areas of anti-diarrheal effects and improved digestion of lactose in lactose-intolerant people, because these findings have been substantiated in human studies. Therefore, some future studies should be performed to use these isolates reliably including:

- Molecular techniques like 16S rRNA sequencing for accurate identification of *Bifidobacterial* species.
- Multiplex RAPD-PCR technique could be used to reveal the complete metabolic potential of each of the probiotic strains.
- Finally animal trial of the strains as probiotics.

These may open an avenue of future research for better efficacy and advancement of food biotechnological research in the food and dairy industries.

Conflict of interest

None to declare

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