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Annals of Applied Microbiology & Biotechnology Journal

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Research Article

Isolation and Screening of Novel Isolates of Bifidobacteria from Human Milk as Potential Probiotic with Antidiarrheal Activity

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Abstract

Aims: The objectives of this research work were isolation of Bifidobacteria from the human milk and its Probiotic characterization such as low pH, bile and *in-vitro* antimicrobial activity against diarrhea causing pathogen.

Methodology and Results: In this research work, 47 bifidobacterial isolates were isolated from the human milk of the 50 lactating women and identified by using phenotypic methods. The isolates were examined *in-vitro* for their tolerance to unfavorable condition at low pH of 2 and 4 and at different concentrations of bile 0.3%, 0.5% and 1%. Further the isolates were tested for the antimicrobial activities by using diarrhea causing indicator stains such as *E. coli*, *Salmonella enterica* and *Shigella boydii*. Antibiotic susceptibility test was performed for the isolates which showed zone of inhibition in antimicrobial testing. Based on the result of *in-vitro* Probiotic test, the best four isolates Dbs18, Smk9, Smk4 and Smk5 were selected for further evaluation of tolerance test of phenol (0.1%, 0.2%, 0.4%), NaCl (5%, 8%, 12%). Auto aggregation and hydrophobicity assay were also done for the four selected isolates. In *in-vitro* test of low pH, out of 47 isolates only 14 isolates were able to grow whereas in bile tolerance assay most of the isolates grew well at 0.3% bile concentration but variability of growth of isolates were observed at 0.5% and 1% bile. In antimicrobial assay, 15 isolates out of 47 isolates showed antimicrobial activity after ruling out the inhibitory activity of low pH. In NaCl and phenol tolerance test all the four selected isolates were able to survive the different concentration of phenol and NaCl. The percentage of hydrophobicity and auto aggregation was highest in Dbs18 followed by Smk9 among the four isolates.

Conclusion, significance and impact of study: Among the four isolates Dbs18 and Smk9 showed good hydrophobicity and auto aggregation ability. These bifidobacterial isolates Dbs18 and Smk9 are found to possess desirable Probiotic properties and will be selected for the *in-vivo* test and molecular identification will be done for the selected isolates. These bifidobacterial strains may act as a potential candidate of novel Probiotic strain isolated from human milk for the treatment of bacterial gastrointestinal diarrhea.

Introduction

Breast milk is the superior food for infants as it fulfills all the nutritional requirements for new born baby. It contains the right balance of nutrients for the growth and development of newborns and also contains many bioactive substances such as IgG, IgM, IL-6 etc that benefit neonates' immune system [1-3]. Besides nutritious the human milk contain beneficial bifidobacterium such as *B. breve*, *B. adolescentis*, *B. longum*, *B. bifidum*, and *B. dentium* which help to protect the baby against various infections and diseases [4]. Breast milk contains prebiotic substances which stimulate the growth of the beneficial bacteria in neonate gut [4]. They are more numerous in the infant gut, where they form up to 91% of the total microflora in breast-fed babies being supported by bifidogenic factors presented in human milk and up to 75% in formula-fed infants [5].

It was reported that the intestinal tract of infants is sterile but gets inoculated during delivery with bacteria of the maternal fecal and vaginal flora and the surrounding environment before birth [6,7]. Bifidobacteria are among the first colonizers of the sterile Gastrointestinal Tract (GIT) of newborns [8] and are one of the predominant groups of the colonic microflora of breast-fed infants [9]. Breast-fed infants show a predominance of Bifidobacteria and lactobacilli, whereas formula-fed infants develop a mixed microbiota with a lower number of Bifidobacteria [10].

Bifidobacteria are gram-positive, anaerobic, catalase negative rods of various shapes (short, regular, thin cells with pointed ends, coccoidal regular cells, long cells with slight bends or protuberances) or a variety of branching (pointed, slightly bifurcated, club-shaped or spatulated extremities), single or chains of various arrangements (in star-like aggregates or disposed in "V" or "Y" or else "palisade" arrangements [11]. Their genome GC content varies from 42 mol% to 62 mol% [12]. Bifidobacterium have several beneficial effects on the health of host such as immunomodulation, elimination of procarcinogens, production of vitamin, prevention of diarrhea and intestinal infections, alleviation of constipation, production of antimicrobials against harmful

intestinal bacteria and protection of the mucosal epithelium against invasion by pathogenic bacteria [13-15].

Diarrhea is still a major worldwide problem and continues to be a major cause of morbidity and mortality in developing countries. According to the report of the World Health Organization (WHO), diarrhea is the second leading cause of death in children under 5 years old which kills around 1.5 million every year. Diarrhea is the third most common cause of death in under-five children, responsible for 13% death in this age-group, killing an estimated 300,000 children in India each year [16]. In the last few decades, several enteric bacteria (e.g., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Clostridium difficile*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *E. coli*) and parasites (e.g., *Cryptosporidium* spp.) have been identified as important causes of diarrhea in human, particularly in infants [17].

Probiotics are considered as functional food since they provide positive health advantages to host when ingested in certain amount. Recently there have been increasing evidences of probiotics in effective prevention of diarrhea. Past decades have witnessed the applications of probiotics in the prevention and management of gastrointestinal disorders [18]. In developing countries like India where the incidence of infectious diarrhea is very high due to poor sanitation and nutrition, the use of Probiotic would be beneficial for public as it is safe, inexpensive and effective.

A number of requirements have to be fulfilled by strains to be an effective Probiotic. The strain should be of human origin for human application, the microbes must survive through the gastrointestinal tract that is they should survive both the stomach and bile acid and should have antimicrobial activities against pathogen [19]. The Probiotic potential of different bacterial strains even within the same species differs. Different strains of the same species are always unique. Therefore, evaluation of Probiotic attributes of bacterial strain is necessary for the selection of Probiotic.

Breast milk is a good natural source of potentially Probiotic or biotherapeutic Bifidobacteria. Bifidobacteria from human breast milk would be a novel indigenous Probiotic of human origin which would be good for the human consumption. However till date, limited work has been carried out on the Probiotic characterization of Bifidobacteria from the human milk as a potential therapy of bacterial gastroenteritis. Therefore, the aim of the current research work was to isolate and characterize Bifidobacteria from the human milk with Antidiarrheal activity.

Materials and Methods

Bacterial strains and culture condition

Test stains *E. coli* (MTCC 9537), *Shigella boydii* (MTCC 11947) and *Salmonella enterica* (MTCC 3858, NTCC 6017) were procured from the Microbial Type Culture Collection (MTCC) of CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India and the strains were cultured in nutrient both. Bifidobacterium stains, *Bifidobacteium bifidum* (NCDC 231) and *Bifidobacteium adolescentis* (NCDC 236) were obtained from the National Dairy Research Institute (NDRI, Karnal) and cultured in de Mann Rogosa Sharpe [20] broth with cystein (MRS-Cys) and were subculture before use. Commercial Probiotic drink Yakult which contains *Lactobacillus casei shirota* was purchased from the market. NCDC 231, NCDC236

and Yakult were included in this study as reference for identification and comparison in *in-vitro* test.

Subjects and sample collection

A total of 50 lactating women who are in the age group of 21 to 37 years were participated in the study based on the following criteria (i) healthy women without any health complication (ii) full term pregnancy (iii) 3-7 days after delivery (iv) not taking antibiotics at the time of sampling. Sampling was approved by the Ethical Committee of Shri Mahant Indresh Hospital, Dehradun, India. The participants provided their milk samples between days 3-7 after delivery. The milk samples were collected by manual expression using sterile gloves. The nipples and mammary areola were cleaned with water then with cotton soaked in chlorhexidine, the first few drops of milk were discarded and milk were collected in a sterile container and kept at 4°C until delivery to laboratory. Analysis of samples took place within one hour of samples collection.

Isolation

Pour plate technique was used to isolate the Bifidobacteria in which 1 ml quantity of milk samples were diluted to 10^{-1}-10^{-4} using sterile peptone water and 0.1 ml quantity of diluted milk samples were pour plated in triplicate onto two different media, MRS medium [20] supplemented with L-cysteine (0.5 g/L) (MRS-Cys) to lower the oxidoreduction potential in culture media and nalidixic acid (2 mg/L) and bifidoselective media, BSC propionate agar base (Hi media, India) respectively for the isolation of Bifidobacteria and the petriplates were kept undisturbed for sometimes for solidification and incubated in an anaerobic jar by using anaerobic gas pack (Hi media, India) at 37°C for 48 hours. After incubation, isolated colonies were randomly picked up from the MRS-Cys and BSC plates of highest dilution and transferred into the sterile (MRS-Cys) broth and purified the selected colonies twice by streaking on the (MRS-Cys) agar. The isolates were maintained in MRS-Cys agar slant and stored at 5-7°C in the refrigerator for short duration.

Identification

Bifidobacteium bifidum (NCDC 231) and *Bifidobacteium adolescentis* (NCDC 236) were used for reference in the identification. The isolates were subjected to primary screening such as gram reaction, cell morphology, non-motility test, catalase test, CO_2 production from glucose, spore test and biochemical test such as indole test, nitrate reduction test and urease test. The isolates which showed Bifidobacteria speciation were selected and preserved in (MRS-Cys) broth with 15% (v/v) glycerol at -20°C for further examination. Cultures were activated prior to experiment by sub culturing twice in the (MRS-Cys) broth.

Tolerance to low pH

Tolerance to low pH was determined by using broth assay. 40μl (2% [vol/vol]) activated overnight culture of the isolates were incubated in 2 ml of MRS-Cys broth in triplicate which was adjusted to pH 2 and pH 4 with 1N HCl or 1N NaOH. The inoculated tubes were incubated for 48 to 72 hour in anaerobic jar by using anaerobic gas pack (Hi media, India) at 37°C and the tubes were observed for turbidity and the cultures which showed turbidity were monitored by determination of optical density at 600 nm in the UV-Vis spectrophotometer (Erba Manheim, Germany).

Bile tolerance

The isolates were determined for their ability to grow in the presence of oxgall by using modified method described by Dora and Glenn [21]. In this assay (2% [vol/vol]) overnight culture of the isolates were inoculated in MRS-Cys broth supplemented with different concentration of oxgall (0.3%, 0.5% and 1% [w/v]). The pH was adjusted to 6 with 1N HCl or 1N NaOH and incubated for 48-72 hour at 37°C an aerobically and the bacterial growth was monitored by determination of optical density at 600 nm in the UV-Vis spectrophotometer.

Antimicrobial Activity

Preparation of sample filtrate

The isolates of Bifidobacteria were inoculated in MRS-Cys media and kept in anaerobic condition in anaerobic jar at 37°C for 48 hour. The overnight cultures were centrifuged at 5000 rpm for 15 min and the supernatants were collected and filtered through 0.22 μm filter.

Antimicrobial activity by disk diffusion method

The sample disks of about 5 mm in diameter were cut from whatman filter paper no. 1 by using punching machine. The disks were put in a petriplate and sterilized by autoclave at 121°C i.e. 15 lbs for 15 minutes and dried in the oven. The indicator strains were inoculated in nutrient broth and incubated at 37°C for 24 hour. The indicator strain was pour plated in the agar media and evenly spread over by glass spreader. The blank disks dipped in the producer strains were kept on the agar plates and pressed slightly with the sterilized forceps. Then the plates were kept at 4°C for diffusion and incubated at 37°C for 24 hour and zone of inhibition were measured in mm. Positive results were recorded when the zone of inhibition is 1 mm around the wells was observed. The experiment was conducted thrice.

Screening of antimicrobial substance

The bacterial isolates which showed antimicrobial activities against the indicator strains were evaluated for their antimicrobial substance. The isolates were grown anaerobically at 37°C for 48 hours and centrifuged at 5000 rpm for 15 min and the supernatants were adjusted at pH 7 with 1N NaOH to rule out the inhibitory activity from the organic acid and the supernatants were filtered through 0.22 μm filter.

Antibiotic susceptibility test

The LAB isolates which had antimicrobial properties in the antimicrobial test were further tested for antimicrobial susceptibility by the Kirby-Bauer method [22] after slight modification. The antibiotics disc (Himedia, Mumbai) used were tetracycline (30 μg), vancomycin (30 μg), erythromycin (15 μg), kanamycin (30 μg), penicillin G (10 units), nalidixic acid (30 μg), gentamicin (10 μg), chloramphenicol (30 μg), rifampicin (5 μg), ofloxacin (5 μg), cefotaxime (30 μg), amoxicillin (30 μg), oxacillin (5 μg). Overnight grown culture of the isolates were pour plated on the MRS-Cys agar and spread evenly with the glass spreader and antibiotic disc were placed and firmly pressed with the sterile forceps and incubated overnight anaerobically at 37°C. Zone of inhibition were measured by using Hi-media zone scale. The results were expressed as sensitive(S), intermediate (I) and resistant (R) according to the National Committee for Clinical Laboratory Standards, NCCLS [23].

Phenol and NaCl tolerance test

The phenol and NaCl tests were done for the four promising isolates Smk4 and Smk5, Smk9 and Dbs18. Tolerance to phenol and NaCl were done in MRS-Cys with NaCl concentration [5, 8 and 12% (w/v)], phenol (0.1,0.2,0.5 g /100 ml) and incubated anaerobically for 48 hours at 37 degree and the isolates were observed for turbidity.

Autoaggregation

Autoaggregation assay was done with certain modification according to Del Re [24].The isolates were grown anaerobically for 48 hours and cells were centrifuged at 5000 g for 15 mins. The pellet were washed with Phosphate Buffer Saline (PBS) and resuspended in the same buffer and initial OD at 600 nm was taken. The bacterial suspensions were vortexed and kept at room temperature for 24 hours and final OD was recorded. Autoaggregation percentage was determined using the equation as: $A\% = (A_0 - A_t)/A_0 \times 100$, where A_t represents the absorbance at time t = 24 h and A_0 represent the absorbance at t = 0.

Hydrophobicity

Hydrophobicity test was done according to Kos [25] with modification in which the isolates were grown anaerobically for 48 hours and harvested by centrifugation at 5000 g for 15 min and pellet were washed with PBS and resuspended in the PBS. To 3 ml of bacterial suspension 1 ml of xylene was added and vortex and kept for the separation of the two phases for one hour and absorbance of aqueous phase at 600 nm was measured. Percentage of hydrophobicity was calculated as (1-A after/ A before) ×100.

Statistical analysis

Data of low pH and bile assay were expressed as mean ± SD of triplicates for each sample. Data were analyzed using the one way ANOVA test. Tukey's test for multiple pair wise comparisons was used to test for differences between the groups. Statistical significance was presented at p<0.05.

Results and Discussion

Isolation and identification

In this present research work search for novel Probiotic against bacterial gastroenteritis was attempted by isolating Bifidobacteria from the human breast milk. Therefore milk samples were collected from the 50 lactating women who were in the age group of 21-37 years. However out of 50 milk samples a total number of 50 isolates were isolated from the 10 milk samples. All the isolates were identified by using conventional methods. Fifty isolates with typical bifidobacterial shape (Figure 1) which are gram-positive, catalase negative, non-spore formation, no CO_2 formation from glucose, unable to produce indole, reduce nitrate and produce urease were selected for further studies. The isolates which showed Bifidobacteria speciation were selected and preserved in (MRS-Cys) broth with 15% (v/v) glycerol at -20°C for further examination. Cultures were activated prior to experiment by sub-culturing twice in the (MRS-Cys) broth. However 3 isolates were lost during the process of sub-culturing and preservation in the MRS-Cys agar slant. It had been found that age of the lactating women did not correlate with the presence or absence of bifidobacterium in their milk as Bifidobacteria were isolated from both the young and old
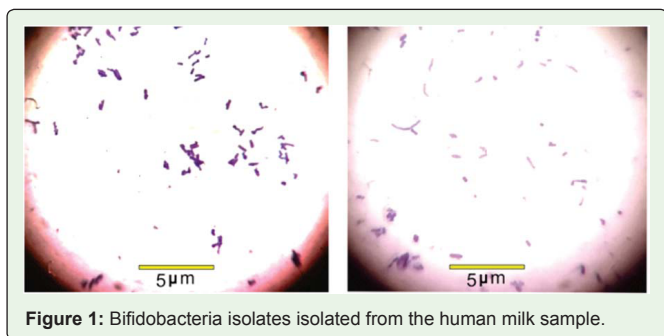
Figure 1: Bifidobacteria isolates isolated from the human milk sample.

Table 1: Bifidobacteria count in human milk sample.

Samples	Age (years)	Total Bifidobacteria counts (c.f.u/ml)
1	29	9.3×10^3
2	27	3.2×10^4
3	21	5×10^3
4	21	5.3×10^3
5	27	8.3×10^3
6	24	1.5×10^4
7	26	7.5×10^3
8	25	1.6×10^4
9	37	4.7×10^4
10	35	3.4×10^4

age lactating women. The present research work also confirmed that Bifidobacteria are indeed present in human breast milk but scanty as Bifidobacteria could be isolated from only 10 lactating women out of 50 (20%). In this study, the viable Bifidobacteria were present in low number (10^4 cfu/ml) in all the milk samples (Table 1). The reason for the less isolation of Bifidobacteria may be attributed to the used of culture dependent methods as there may be Bifidobacteria which are not cultivable or difficult to cultivate. Our findings are in agreement with the findings of Sallam et al. [26] who reported that culture of the breast milk specimens showed growth of lactobacilli in the milk of 50 mothers (100% of the study population) and Bifidobacteria in the milk of 14 mothers (28%). Martín et al. [4] also isolated Bifidobacteria from 8 milk samples out of 23 women (10^3 CFU/ml in breast milk). It is also reported that lactobacilli and Bifidobacteria could be isolated from the milk of 27 (40.91%) and 7 (10.61%), respectively of the 66 cultured samples [27].

Intolerance to low pH and bile

The main criterion for selection of Probiotic strain is to survive the low pH of stomach and bile of intestine. Before reaching the gastrointestinal tract, Probiotic bacteria must first survive transit through the stomach and have their health promoting effects as metabolically viable active cells when they arrive in the colon [28]. In low pH test, the isolates which developed turbidity after 48-72 hours were considered to be viable and optical density was recorded. Out of the 47 isolates, 14 isolates were able to survive the stress factor of low pH (pH 2) up to 48-72 hours of culturing. Significant variations existed among the cultures with regard to their ability to grow at pH 4 (data not shown) and pH 2 (p<0.05) and the isolates, Bds1

was slightly increased significantly than Y at p<0.05 (Figure 2). Our results are in agreement with the previous studies which reported the viability of Bifidobacteria at pH values of gastric juices is considered to be generally low [29-32] as only few Bifidobacteria isolates were able to survive at low pH.

Bile tolerance is more important than tolerance to low pH as bile salt is more detrimental than the effects of low pH and encapsulation can protect the bacteria from the harmful effects of low pH. The most bile-resistant cultures which also possess other desirable characteristics should be selected as a dietary adjunct [33]. In bile tolerance assay, different bile salt concentrations of 0.3%, 0.5% and 1% were used. The effects of growth of the isolates in the presence of different concentration of bile were compared with the absorbance value obtained. Most of the isolates grew well at 0.3% bile but growths of the isolates were varied at p<0.05 (Figure 3). At 0.5% bile, all the isolates did not grow well and at 1% only few isolates were able to grow up to 48-72 hours of culturing. In the human GI-tract, the mean bile salt concentration is believed to be 3000 ppm (0.3%), which is considered as critical and high enough to screen for resistant strains [34,35].

Antimicrobial activity

One of the most important properties of Probiotic is protection against pathogen in the intestinal tract of the host [36]. Diarrheagenic *E. coli* represents a leading bacterial cause of pediatric diarrhea
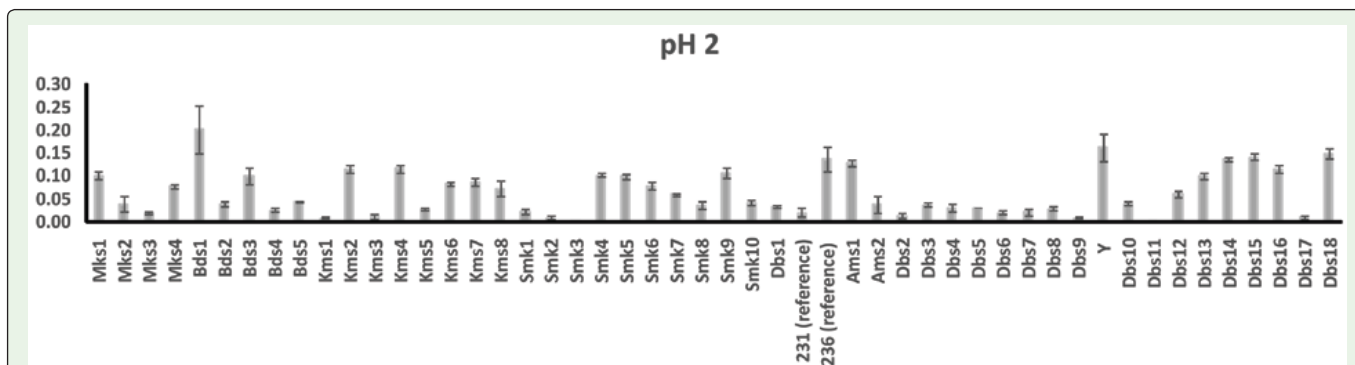

Figure 2: Acid tolerance of the isolates grown in MRS-Cys broth adjusted to pH 2. Columns with standard errors are statistically significant difference at P<0.05, as determined by one-way ANOVA incorporating Tukey's Honestly Significance Difference for multiple pair-wise comparisons. Value at each time point in the line graph is the average of 3 replicate analyses. Y- Yakult (commercial probiotic drink).
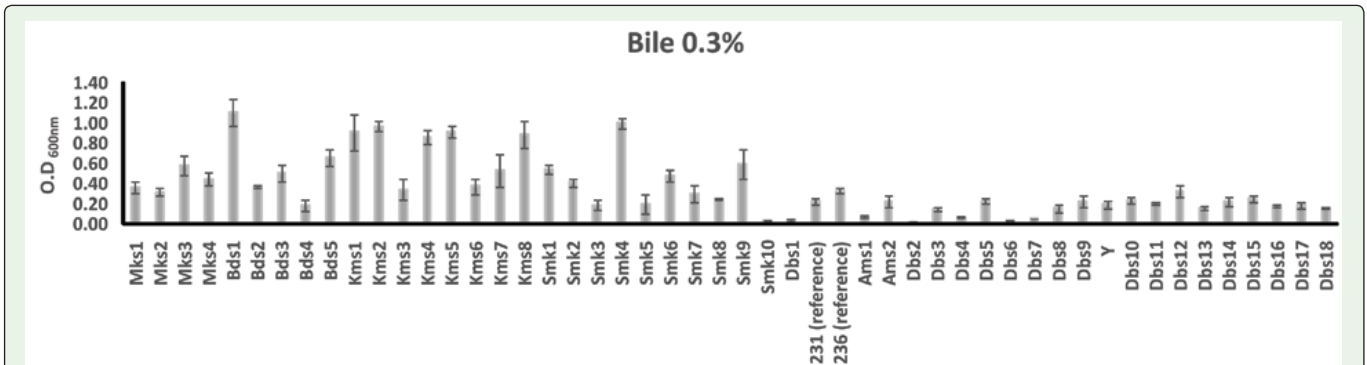
Figure 3: Survival of the isolates in MRS-Cys broth supplemented with 0.3% oxgall. Within a parameter, mean values with standard errors are statistically significant difference at P<0.05, as determined by one-way ANOVA incorporating Tukey's Honestly Significance Difference for multiple pair-wise comparisons. Value at each time point in the line graph is the average of 3 replicate analyses. Y–Yakult (commercial probiotic drink).

in developing countries. Nontyphoid Salmonella is the common pathogen which caused gastrointestinal disease in the world and comprises of *S. enterica*, *S. bongoi* and *S. subterranean* [37]. Shigella are divided into four groups depending on serologic similarity and fermentation reactions: group A (*Shigella dysenteriae*), group B (*Shigella flexneri*), group C (*Shigella boydii*), and group D (*Shigella sonnei*) in which *Shigella boydii* is prevalent in Indian sub-continent. In the present research study, the antagonistic effects of the cell-free filtrates of each of the 47 isolates were evaluated in which 29 isolates were shown to inhibit the test pathogens (Table 2). The zones of inhibition of the isolates which have antibacterial activities were in the range of 6 mm-13 mm which is inclusive of 5 mm disc. According to Schillinger and Lucke [38], zone of inhibition of 0.5-1 mm is

considered as small inhibition zone and >1.0 mm is considered as large inhibition zone. Therefore most of the isolates showed large zone of inhibition (>1 mm) (Figure 4). The bifidobacterial isolates which showed zone of inhibition were selected for the characterization of antimicrobial compound (Table 3). To rule out the acid inhibition, NaOH (1N) were added, after neutralization of the CFF only 15 isolates showed zone of inhibition which could be due to hydrogen peroxide or bacteriocin. Thus, the result showed that not all the Bifidobacteria present in the breast milk have antimicrobial property and the isolates Smk4 and Smk5, Smk9 and Dbs18 showed zone of inhibition against all the test pathogens as compare to Y.

Antibiotic susceptibility test

There is concern over the possible spread of antibiotic resistance determinants from bacteria used in Probiotic products. Routine testing of antibiotic susceptibility Lactic Acid Bacteria (LAB) and Bifidobacteria may be advisable for checking the biosafety of potential Probiotic isolates [39]. Antibiotic susceptibility test were screened on the 15 isolates which showed antibacterial activities by using 13 antibiotic discs (Hi media, India). All the isolates were resistant to quinolines group of antibiotic (nalidixic acid) except Ams2 and Dbs16. Most of the isolates were susceptible to β-lactams group of antibiotics (penicillin and oxacillin), 8 isolates were resistant to gentamicin and 10 isolates were resistant to kanamycin. All the isolates were resistant to vancomycin except Dbs16. All the isolates were sensitive to rifampicin, cefotaxime, chloramphenicol, amoxycillin and tetracycline. Ten isolates were resistant to ofloxacin and all the isolates

Table 2: Zone of inhibition of the isolates against the indicator stains.

Isolates	*E. coli* (MTCC 9537)	*Salmonella* (MTCC 3858)	*Shigella* (MTCC 11947)
Kms2	+	-	-
Smk1	+	+	-
Smk3	-	-	+
Smk4	+	+	+
Smk5	+	+	+
Smk7	-	+	+
Smk9	++	+	+
Smk10	-	+	-
Dbs11	-	+	-
Dbs12	-	+	-
Dbs14	+	+	-
Dbs15	+	+	-
Dbs16	+	+	-
Dbs18	+	+	+
Ams2	++	-	-
Y	-	+	+
231	-	+	-
236	+	+	-

Symbols: +: (6-9 mm); ++: (10-13 mm); -: no zone of inhibition; Y: Yakult (commercial probiotic drink); 231 & 236: References

Figure 4: Antimicrobial activity of the isolates against the indicator strains (a) MTCC 3858 & (b) MTCC 9573. Y – Yakult (commercial probiotic drink); C-Control.

Table 3: Antimicrobial susceptibility of the Bifidobacterial isolates.

Isolates	NA 30mcg	K 30mcg	RIF 5mcg	VA 30mcg	CT X 30mcg	C 30mcg	AM X 30mcg	TE 30mcg	O X 5mcg	OF 5mcg	P 10units	GEN 10mcg	E 15mcg
Dbs18	0	0	35	0	35	35	35	29	24	0	35	21	40
Dbs15	0	20	22	0	30	26	32	23	25	10	30	15	25
Dbs12	0	24	21	0	30	26	27	35	30	12	35	13	35
Smk9	5	15	38	0	30	21	40	25	0	0	30	12	30
Smk7	6	0	26	0	35	35	35	25	30	8	30	10	25
Smk1	0	0	30	0	30	28	30	23	24	18	34	15	7
Dbs14	0	10	30	0	30	30	30	26	20	19	25	19	33
Dbs11	4	17	21	4	24	29	26	28	11	20	29	20	30
Kms2	0	10	26	0	30	30	35	25	17	20	30	18	25
Smk10	0	15	35	0	25	40	40	30	0	17	20	25	35
Ams2	17	22	20	3	25	33	28	20	14	10	17	20	32
Smk3	8	28	31	2	23	36	25	26	27	14	29	15	24
Dbs16	18	15	30	20	35	34	24	35	34	30	26	25	0
Smk4	0	0	29	0	25	34	35	26	10	12	28	14	30
Smk5	0	0	28	0	27	30	25	24	0	0	10	14	25
Y	30	30	5	30	30	30	30	28	5	5	10	10	15

Symbols: GEN: Gentamicin; TE: Tetracycline; E: Erythromycin; C: Chloramphenicol; P: Penicillin; RIF: Rifampicin; OF: Ofloxacin; CTX: Cefotaxime (Cephotaxime); AMX: Amoxicillin; K: Kanamycin; NA: Nalidixic acid; OX: Oxacillin

except Smk1 and Dbs16 were sensitive to erythromycin. Yazid et al. [40] reported that all tested 18 Bifidobacteria strains from 10 species were sensitive to amoxicillin. Moubareck et al. [41] also reported that all tested strains of Bifidobacteria were sensitive to penicillin G and amoxicillin. Lim et al. [42] reported that tested Bifidobacteria were sensitive to erythromycin, most resistant to kanamycin nalidixic acid, less resistant to gentamicin and susceptibility to tetracycline was variable. Charteris et al. [29] observed vancomycin resistance as a general characteristic for Bifidobacteria by using a disc diffusion method.

Phenol and NaCl test

Tolerance to phenol is a characteristic Probiotic property because phenols can be formed in the intestines by bacteria that deaminate some aromatic amino acids delivered by the diet or produced by endogenous proteins [33]. In the phenol test the four selected isolates Smk4 Smk5, Smk9 and Dbs18 were able to grow up to 0.4%. In NaCl test all the four isolates could tolerate up to 12% NaCl. Ability to grow at high salt concentration is important for Probiotic as they can inhibit the growth of pathogen (Table 4).

Cell surface properties

Auto-aggregation determines the ability of a bacterial strain to interact with itself, in a nonspecific way, which is known as a prerequisite for colonization and infection of the gastrointestinal tract by pathogens through adhesive ability [24]. The rate of autoaggregation was measured for all the four isolates after 24 hours and the result showed that all the isolates showed more than 40% which were higher than Y in which Dbs18 had the highest rate of autoaggregation 70% followed by Smk9 47.9%. Results are expressed as percentage reduction after 24 hours (Figure 5a). Yong et al. [43] reported that the percentage of autoaggregation of *Bifidobacteria animalis* subsp.
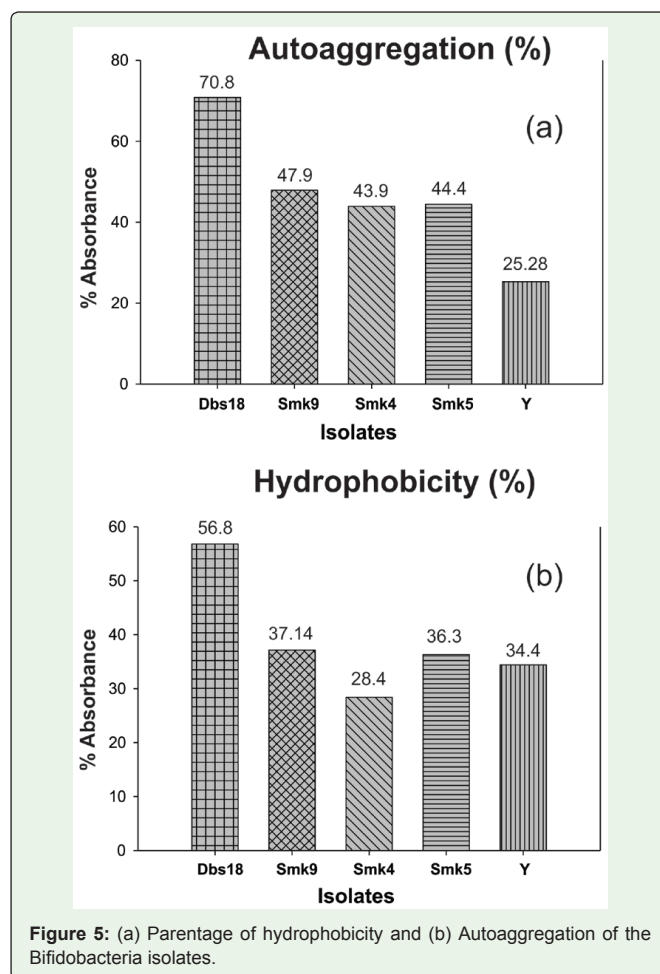

Figure 5: (a) Parentage of hydrophobicity and (b) Autoaggregation of the Bifidobacteria isolates.

Table 4: Phenol and NaCl tolerance test of the selected isolates of Bifidobacteria (OD at 600nm).

Isolates	Phenol			NaCl		
	0.1%	0.2%	0.4%	5%	8%	12%
Dbs18	2.105 ± 0.10	1.441 ± 0.47	0.328 ± 0.042	1.802 ± 0.05	0.149 ± 0.028	0.119 ± 0.023
Smk9	1.427 ± 0.40	1.193 ± 0.53	0.504 ± 0.146	1.369 ± 0.228	0.274 ± 0.146	0.151 ± 0.038
Smk4	1.033 ± 0.39	0.947 ± 0.097	0.32 ± 0.049	1.466 ± 0.209	0.284 ± 0.076	0.131 ± 0.015
Smk5	1.438 ± 0.45	1.38 ± 0.543	0.319 ± 0.106	1.454 ± 0.112	0.335 ± 0.155	0.164 ± 0.049
Y	1.657 ± 0.19	1.25 ± 0.33	0.481 ± 0.02	1.136 ± 0.369	0.244 ± 0.039	0.176 ± 0.029

lactic BB12 and *Lactobacillus casei* Shirota were respectively 36.7% and17.9%. Our results showed that the rate of autoaggregation of the isolates were above the acceptable value (>40%) as referred by Del Re et al. [44]. The result of cell surface hydrophobicity showed that the Dbs18 had strongest hydrophobicity ability of 56.8% to xylene (a polar solvent) while others isolates revealed low percentage. Results of hydrophobicity are expressed as percentage reduction after 1 hour (Figure 5b). The probable reason of low percentage could be low affinity of the isolates to polar solvent [45].

Conclusion

It can be concluded that the result of this research work confirmed the presence of Bifidobacteria in human breast milk but scanty. The desirable Probiotic attributes were not present in a single isolate. However, some of the isolates of Bifidobacteria showed promising ability to grow at low pH 2 and bile concentration of 0.5% and 1% and showed antimicrobial activity against pathogens causing diarrhea. The best four isolates Smk4, Smk5, Smk9 and Dbs18 were also and able to tolerate the high NaCl and phenol concentration. Among the four isolates Dbs18 and Smk9 showed good hydrophobicity and auto aggregation ability. In our present investigation the isolates Dbs18 and Smk9 were found to possess desirable Probiotic properties and will be selected for the *in-vivo* colonization test. These bifidobacterial strains may act as a potential candidate of novel Probiotic strain isolated from human milk for the treatment of bacterial gastrointestinal diarrhea.

Acknowledgment

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