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PROBIOTIC ATTRIBUTES OF THE NEWLY ISOLATED LACTIC ACID BACTERIA FROM INFANTS'GUT

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ABSTRACT

The gut microbiota intimately interacts with the host and influences physiological, metabolic, genetic, and immunological attributes. In the current study, analysis of fecal samples from healthy breast fed infants showed that lactic acid bacteria constitute the most abundant bacterial spp. in the infant gut (*Lactobacillus plantarum* being the most predominant) contrary to the established reports that *Bifidobacterium* spp. are mostly predominant in the infants' gut. Evaluation of lactic acid bacteria (LAB) isolates for important probiotic characteristics showed that several of the isolates were well equipped with desirable probiotic attribute viz. ability to grow at the gut pH, and at high NaCl, bile and phenol concentrations. Some of the LAB isolates expressed functional properties like high hydrophobicity, cholesterol lowering ability, exopolysacharide producing ability, and antimicrobial potential against human pathogens. Three LAB isolates found to be superlative on probiotics attributes were identified as *Enterococcus faecium* FS-1.1, *E. faecium* FS-2.0, and *E. faecium* FS-4.0, based on 16S rDNA sequence analysis. Furthermore, the fecal samples of breast fed infants showed lower level of β-glucuronidase and higher level of β-glucosidase as compared to the formula fed infants, thus, reflecting the importance of breast-feeding towards general health and well being of infants.

Keywords: Lactic acid bacteria, breast/formula fed infants, hydrophobicity, exopolysaccharide, β- glucuronidase, β-glucosidase

INTRODUCTION

The microbiota of a newborn develops rapidly, and is strongly dependent on the mother's microbiota, mode of birth, physical environment and hygiene during birth, and subsequently influenced by feeding practices and rearing environment of the infant (**Salminen *et al.*, 2005; Ozen and Dinleyici, 2015**). The bacteria which are found during the various stages of colonization include members of the genera *Bifidobacterium*, *Enterococcus*, *Clostridium* and *Enterobacter*, among others (**Weng and Walker, 2006; Bajaj *et al.*, 2015**). Bifidobacteria constitute the largest group of bacteria in the intestine of infants, however, their number actually declines in the human body with age (**Ishibashi *et al.*, 1997**). In breast-fed infants bifidobacteria constitute about 90% of their intestinal bacteria; however, this number is lower in formula-fed infants (**Zinedine and Faid, 2007**). When breast-fed infants' diet is changed to cow's milk and solid food, bifidobacteria are joined by other bacteria found in the human body such as *Bacteroides*, streptococci and lactobacilli, and others. Lower number of bifidobacteria in formula-fed infants attributes towards a higher risk of diarrhea and allergies that is usually associated with babies who aren't breast-fed. In addition, because bifidobacteria produce lactic acid instead of gas (unlike *E. coli)*, infants and people in general with more bifidobacterial population than other bacteria may have less gas and digestive problems (**Hussey *et al.*, 2011**). The characterization of fecal enzymes has been used as an indicator for the gut microflora composition, and its association with hosts' health status, suggesting that gut microbiota is stout and unified system (**Mc Intosh *et al.*, 2012**).

The foremost endeavor of the current investigation was to study the dominant bacteria in the breast-fed infants born out of the vaginal mode of delivery, and to characterize the probiotic potential of the isolates from infant feces; in addition fecal enzyme status of the infants based upon difference in the age span and feeding habits was also investigated. Probiotics are live microorganisms (single/mixed cultures) which impart multiple health benefits to the consumer animals or humans. Probiotics include different lactic acid bacteria viz. *Lactobacillus* spp., *Bifidobacterium*, and the yeast *Saccharomyces boulardii*, among several others. The selection of probiotic strains from appropriate sources depending on the target population, such as neonates and children whose gut microbiota may differ from that of healthy adults, constitutes a promising approach (**O'Toole and Claesson, 2010**). In this regard most of the strains currently available have targeted the adult population but fewer strains have been selected for other groups of age, such as human neonates, where the applications of probiotics may have a great positive impact (**O'Toole and Claesson, 2010**). Demand of probiotics has increased in recent years due to enhanced availability of evidences for health benefits. Isolation of novel strains of probiotics with proficient health benefitting characteristics has gained immense research impetus considering that health benefits of probiotics(s) cannot be generalized i.e. health attributes earmarked in one strain or species may not be necessarily present in other members of genus/species or strains (**Bajaj *et al.*, 2014; Bajaj *et al.*, 2015**). Therefore, bioprospecting of novel probiotics strains from unexplored ecological niches would be advantageous for targeting novel strains with potential functional characteristics for future applications in food/pharmaceutical industries. In addition, this practice may help investigating taxonomic characteristics of microbial isolates/strains for potentially novel biotechnological applications. Sources for isolation of probiotics could be immensely diverse e.g. traditional fermented foods, plant, animal, human and marine sources. Proficient probiotic(s) must possess certain desired characteristics like ability to survive in gut, adhesion ability in intestine, antagonistic potential against pathogens, devoid of antibiotic resistance, exopolysaccharide producing ability, among others. Furthermore, should a single organism not possess all such desired features, a combination may be used as probiotics. Current article describes the recent developments in the area of bioprospecting of probiotics (**Bajaj *et al.*, 2014**).

Gastrointestinal (GI) tract infections constitute one of the major causes of morbidity and mortality among infants/children worldwide. Every year around 1,575,000 children under the age of five die due to severe diarrhea and dehydration representing 15% of the 10.5 million deaths per year of children in this age group (**Lopez *et al.*, 2006**). Certain probiotics are found to be helpful in preventing and treating some types of bacteria-induced diarrhea because of their ability to alter the activity of the intestinal microflora and compete with the potential pathogens (**Marteau *et al.*, 2001; Bajaj *et al.*, 2015**). Therefore, targeting of probiotics specific for infants/children has been emphasized. Furthermore, the quest for novel efficacious probiotic strains is a continuous process, keeping in view the huge diversity among probiotic strains with respect to their health augmenting attributes. Thus, new probiotic strains with appropriate properties may be identified for potential infant applications.

MATERIALS AND METHODS

Sample collection isolation and identification of bacteria

Fecal samples of healthy infants borne out of vaginal course of delivery were collected from the Department of Paediatrics, Government Medical College and Allied Hospitals, Jammu, in sterile sample containers and maintained at 4°C and assayed within an hour or two of collection. The consent of the parents of the infants was taken before the samples were collected and the medical history of the infant and the mother was documented. It was ensured that both the mother and the infant had not taken any antibiotic for last 2 weeks.

One gram of fecal sample was suspended in saline (0.85% w/v, NaCl), serially diluted, and spread plated on MRS agar supplemented with L- cysteine hydrochloride (0.05%, w/v) and gentamycin (100mg/L). Plates were incubated in an anaerobic chamber (HiMedia Laboratories Pvt. Ltd, India), at 37°C for 48-72 h. The colonies obtained were purified on MRS agar and examined microscopically. The selected isolates were maintained in skimmed milk (10%, w/v) at 4°C for smaller duration, and at -20°C in skimmed milk supplemented with glycerol (50%, w/v) for long term storage.

The isolates were studied for Gram reaction, catalase test, spore staining, and growth at 10°C and 45°C for 48 h, and production of CO_2 from glucose (**Patil *et al.*, 2010**). β-galactosidase and fructose 6-phosphate phosphoketolase activity (F6PPK) tests were done (**Martnez *et al*., 2012**) for the preliminary examination of lactic acid bacteria (LAB). The ability of LAB to ferment various carbohydrates was examined using HiCarbohydrate™ kit (HiMedia Laboratories Pvt. Ltd, India). Interpretation of fermentation profiles of carbohydrates was done using the PIBWin software, which is the ID tool used to run the Ashex matrix. Ashex is an enormous system of excel worksheets, formed by analyzing and compiling data from hundreds of biochemical tests (http://www.som.soton.ac.uk/staff/tnb/pib.htm).

The selected LAB isolates were identified based upon 16S rDNA sequence analysis. DNA was isolated (Hi Media DNA extraction kit) and PCR-amplified using the universal primers: forward, lac1-27F 5'-AGAGTTTGATCCTGGCTCAG and reverse, lac 1-1492R 5'TACGGYTACCTTGTTACGACT (IDT/Promega) in a thermocycler (Eppendorf, Mastercycler gradient). PCR reaction was run for 30 cycles. The following thermal profile was used for the PCR: denaturation at 94°C for 4 min, primer annealing at 52°C for 45 sec and extension at 72°C for 1 min and 30 sec. The final cycle included extension for 10 min at 72°C. The amplified product the (~1,500 bases) was sequenced (Department of Biochemistry, University of Delhi, South Campus). The sequence was subjected to Mega Blast analysis from Genbank data base of NCBI, and dendrogram generated (Mega5).

Growth studies at different pH, and in presence of NaCl, bile salt and phenol

For assessing the ability of isolated LAB to grow over (and tolerate) wide range of pH (2, 4 and 8), NaCl (4, 8 and 12 %, w/v), bile salts (2 and 4%, w/v) and phenol (0.2, 0.4 and 0.6%, w/v), the MRS broth used for growth studies was added with corresponding amounts of inhibitory substances viz. NaCl, bile salts or phenol or adjusted at desired pH. The LAB cultures were activated by growing them in MRS broth for 18 h (A_{600}, 0.8) and then inoculated (@1 %, v/v) into different MRS broth having varying pH, NaCl, bile salts or phenol concentration. Incubation at 37˚C under static conditions was given for 24h and growth was measured spectrophotometerically at 660 nm (UV-VIS 1800, Shimadzu, Japan). Growth in plain MRS broth at pH 6.5 served as control.

Hydrophobicity of the LAB isolates

Cell surface hydrophobicity was determined based on the method described by (**Aswathy *et al*., 2008**) with slight modification. LAB isolates were grown in MRS broth, centrifuged (7500 ×g for 5 min, Sigma, 3K30) and the cell pellet was washed thrice with ringer solution (each w/v, NaCl 6%, KCl 0.0075%, $CaCl_2$ 0.01% and $NaHC0_3$ 0.01%) and suspended in the ringer solution and examined for absorbance at 580 nm. Cell suspension was mixed with n-hexadecane (3:1) in a cyclomixer for 2 min and allowed to stand for 30 min to get the two phases separated. The lower phase was examined spectrophotometrically (580 nm). The percent hydrophobicity of strain adhering to n-hexadecane was calculated using the following equation:

$$\text{Percent hydrophobicity} = \frac{A_{580}\,(\text{bm}) - A_{580}\,(\text{wh})}{A_{580}\,(\text{bm})}\; x\; 100$$

Where: bm – before mixing, wh – with hexadecane

Antibacterial activity of LAB isolates

For determining antibacterial activity, the isolates were grown in MRS broth for 18 h at 37˚C under static conditions. Cultural broth was centrifuged (8000×g for 5 min) and the supernatant was filtered through a bacterial filter (Whatman, 0.22 µ). The filtrate was used for assaying the antimicrobial activity after adjusting the pH to 6.4. The test organisms (*Bacillus subtilis*, *B. cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *P. alkaligenes*, *Staphylococcus aureus* and *Streptococcus* sp., procured from culture collection of Fermentation Biotechnology Lab. of School of Biotechnology, University of Jammu, Jammu) were grown for 18 h at 37ºC in Luria-Bertani broth (A_{600} 0.6) and spread plated on Mueller-Hinton agar. The wells (6 mm each) were cut on the lawn of spread culture and the cultural filtrate obtained above was poured (@45 µl) into each well, allowed the filtrate to diffuse for 2-3 h, and then plates were incubated for 24-48 h at 37˚C. The appearance of zone of inhibition around the well was suggestive of antimicrobial activity and the same was measured.

Cholesterol-lowering ability of LAB isolates

MRS broth containing L-cysteine hydrochloride (0.05%, w/v) was supplemented with pre-filtered (0.45µm Whatmann) soluble cholesterol (polyoxyethanyl-cholesterol sebacate, Sigma, USA) and inoculated with LAB culture to attain final concentration of 10^8~10^9 CFU/ ml of solution. Incubation was given anaerobically at 37°C for 24 h. After incubation the cultures were centrifuged (8000 g for 10 min), and the supernatant was assayed for residual cholesterol content (**Rudel and Morris, 1973**) Uninoculated cholesterol-MRS broth served as the control.

Exopolysaccharides (EPS) production by LAB isolates

EPS producing ability of LAB was assessed (**Frengovaa *et al*., 2002**). Selected isolates were grown overnight in modified MRS broth (glucose and lactose, each @10%, w/v) for 37 °C and centrifuged (11000 ×g for 10 min) to obtain the supernatant. Two volumes of cold ethanol was added to the supernatant and stored overnight at 4°C. Precipitated material was collected by centrifugation (2500×g for 20 min) resuspended in demineralised water and re-precipitated by mixing with 2 volumes of cold ethanol, and allowed to stand for 12-18 h. The precipitate was separated by centrifugation (2500×g for 20 min), pellets were air dried and assayed for total carbohydrate content using the phenol-sulfuric acid procedure (**Dubois *et al*., 1956**).

Fecal analysis of enzyme (β-glucuronidase and β-glucosidase)

One gram of fecal sample was diluted in phosphate buffered saline and used as an equivalent to crude enzyme preparation for analysis. β-glucuronidase (EC 3.2.1.31) was assayed by using phenolphthalein-β-D-glucopyranosiduronic acid as substrate (in 0.1 mol/L of potassium phosphate, pH 6.8), and β-glucosidase (EC 3.2.1.21) was assayed by using *p*-nitrophenyl-β-D-glucopyranoside as substrate (in 0.1 mol/L potassium phosphate, pH 7.4) by using spectrophotometric method (**Freeman, 1986**). The activity of β-glucuronidase and β-glucosidase was expressed in terms of amount of products i.e. phenolphthalein and *p*-nitrophenol, respectively, generated (millimoles) from their respective substrates, per h per mg of protein. All analytical experiments were run in triplicate, and data presented is the mean of three values.

RESULTS

Isolation and biochemical analysis of LAB/bifidobacteria from infant fecal samples

A total of 50 infant fecal samples (exclusively breast fed infants and borne by vaginal mode of delivery) were used for isolation of bacteria. The samples were enriched in MRS broth and a total of 135 bacterial isolates were obtained on the MRS agar plates. All the isolates were purified and maintained on skimmed milk for further studies. Based upon biochemical and physiological analysis of a total of 93 bacterial isolates, 77 of the isolates were identified as LAB (82%) while 16 (approximately 18%) isolates were earmarked as *Bifidobacterium* sp. (**Zinedine and Faid, 2007**) as shown in Table 1. Among lactobacilli *Lactobacillus plantarum* was the most predominating sp. (18 isolates), and was followed by *L. casei* subsp. *casei* and *L. brevis* (6 isolates, each), *L. casei* (4 isolates), *L. fermentum* (3 isolates) and *L. helveticus* (2 isolate each); one isolate each of *L. casei* subsp. *alactosus*, *L. bulgaricus* and *L. viridescens* was reported. Among the enterococci *E. faecium* was found to be the predominant (14 isolates), followed by *E. durans* (11 isolates) and *E. faecalis* (10 isolates).

Table 1 Lactic acid bacteria and bifidobacteria isolates from infant fecal samples

Infant fecal isolates	Number of isolates	Identification
FS1.2, FS 3.2, FS 4.4, FS 4.7, FS 5.2, FS 11.4, FS12.1, FS14.1, FS14.4, FS17.2, FS 29.3, FS 6.3, FS11.2, FS15.1, FS20.1, FS 23.2, FS 29.4, FS 31.3	18	*Lactobacillus plantarum*
FS 9.1, FS 10.4, FS 20.4, FS 17.1, FS 28.1, 29.1.	6	*Lactobacillus brevis*
FS 13.1, FS 16.4, FS 24.1, FS 27.2, FS 25.6, FS 34.3	6	*Lactobacillus casei* subsp. *casei*
FS 12.5, FS 22.2, FS 30.2, FS 32.1	4	*Lactobacillus casei*
FS 15.2, FS 31.1, FS 50.1,	3	*Lactobacillus fermentum*
FS 30.1, FS 12.6,	2	*Lactobacillus helveticus*
FS 33.1	1	*Lactobacillus casei* subsp. *alactosus*
FS 33.2	1	*Lactobacillus bulgaricus*
FS 34.2	1	*Lactobacillus viridescens*
FS 37.1, FS 37.2, FS 38.2, FS 43.1, FS 44.1, FS 46.2, FS 50.2, FS 31.4, FS 47.2, FS 50.3	10	*Enterococcus faecalis*
FS1.1, FS2.0, FS4.0 FS 36.1, FS 38.1, FS 45.3, FS 46.1, FS 49.1, FS 23.1, FS 25.1, FS 43.2, FS 47.1, FS17.3, FS-50.4	14	*Enterococcus faecium*
FS 35.1, FS 39.1, FS 40.2, FS 42.1, FS 42.2, FS 44.3, FS 47.3, FS 23.3, FS 25.3, FS 49.2,FS-6.1	11	*Enterococcus durans*
FS 12.2, FS 13.2, FS 13.3, FS 16.1, FS 31.5, FS 34.1, FS 34.4, FS 38.3, FS 38.4, FS 39.2, FS 39.3, FS 1.3, FS 1.4, FS 1.5, FS 4.2, FS 4.6	16	*Bifidobacterium* spp.

Growth study of LAB/bifidobacteria isolates under (gut like) inhibitory conditions

Among LAB isolates, 11 grew appreciably at pH 2 and 4 (A_{600}, 0.5-0.1), 21 showed average growth (A_{600} 0.1-0.05) while the rest grew relatively slower (A_{600} 0.05 or less). *L. plantarum* FS-1.2, *E. faecium* FS-2.0 and *L. plantarum* FS-4.4 showed maximum growth. Sixty one isolates grew well at 4% NaCl concentration wherein isolate *L. casei* subsp. *casei* FS-13.1 (A_{600} 0.570) and *L. plantarum* FS-3.2 showed maximum growth (A_{600} 0.473); 33 of the LAB isolates showed good growth at 8% NaCl (A_{600}, 0.5-0.1) while only 2 isolates *L. plantarum* FS 12.1 and *L. casei* FS 12.5 grew at 12% NaCl. Sixty seven LAB isolates showed growth (absorbance of 0.1 or above) at 0.2% phenol while only 18 did so at 0.4% of phenol. As the phenol concentration further increased (0.6%) growth of all the isolates declined. *E. faecium* FS-4.0 was the lone isolate which showed appreciable growth at 0.6% of phenol. Sixty isolates grew well at 2% bile while only three isolates (*E. faecium* FS-4.0, *L. casei* FS-32.1 and *E. faecium* FS-38.1) showed appreciable growth at 4% bile concentration. Thus, several of the Lab isolates were able to withstand and grow over varying range of inhibitory agents/conditions generally encountered in the GI environment.

Hydrophobicity of the LAB/bifidobacteria isolates

All LAB and bifidobacteria isolates were evaluated for the degree of hydrophobicity in order to examine their adhesion property in the gut. The bacterial isolates displayed varying level of hydrophobicity (Table 2). The isolate *L. plantarum* FS 1.2 showed the maximum hydrophobicity (92.6%) and was followed by *Bifidobacterium* sp. FS 4.6 (90.7%), *E. faecium* FS 46.1 (90.2%), *L. viridescens* FS 34.2 (87.2%), *L. helveticus* FS 30.1 (84.0%), *L. fermentum* FS 15.2 (83.4%), *L. plantarum* FS 3.2 (82.1%), *L. brevis* FS 29.1 (82.0%) and *E. faecium* FS 1.1 (80.1%). Forty six of the LAB isolates had hydrophobicity in the range of 50-80% while rest of the isolates displayed hydrophobicity that was less than 50%.

Table 2 Functional attributes (hydrophobicity, EPS producing, and cholesterol-lowering ability) of probiotic lactic acid bacteria isolates from infant fecal samples

LAB isolates	Hydrophobicity (%)	LAB isolates	EPS (mg/l)	LAB isolates	cholesterol reduction (%)
L. plantarum FS 1.2	92.6±0.25	*L. plantarum* FS 1.2	814±11.51	*L. plantarum* FS 1.2	59.6±1.25
E. faecium FS 46.1	90.2±0.15	*E. durans* FS 6.1	647 ±13.22	*L. plantarum* FS 4.7	58.09±0.8.5
L. viridescens FS 34.2	87.2±0.02	*L. casei* subsp. *casei* FS 25.6	619±14.54	*E. faecalis* FS 37.1	58.02±0.92
L. helveticus FS 30.1	84.0±0.35	*E. faecalis* FS 50.1	616±12.42	*L. casei* FS 12.5	57.65±1.12
L. fermentum FS 15.2	83.4±0.22	*E. durans* FS 39.1	570±13.73	*L. helveticus* FS 12.6	57.02±1.48
L. plantarum FS 3.2	82.1±0.55	*L. casei subsp casei* FS 27.2	550±9.91	*E. faecalis* FS 37.2	56.20±1.32
L. brevis FS 29.1	82.0±0.11	*L casei* FS 22.2	523±14.96	*L. casei* FS 22.2	54.42±0.65
E. faecium FS 1.1	80.1±0.17	*L. casei* subsp *casei* FS 13.1	512±11.11	*L. casei* subsp. *casei* FS 25.6	53.36±1. 51
E. durans FS 39.1	79.1±0.45	*E. faecalis* FS 38.2	512±8.59	*L. plantarum* FS 14.1	53.20±0.87
L. plantarum FS 33.1	78.5±0.63	*E. faecium* FS 2.0	510±6.67	*L. helveticus* FS 30.1	52.94±1.05
L. brevis FS-10.4	78.3±0.78	*L. fermentum* FS 15.2	509±10.14	*L. plantarum* FS-6.3	49.79±1.81

EPS producing ability of the LAB and bifidobacteria isolates

Various LAB isolates showed varying level of EPS production**.** *L. plantarum* FS 1.2 showed maximum level of EPS production (814 mg/l), and was followed by *E. durans* FS 6.1 (647 mg/l), *L. casei* subsp. *casei* FS 25.6 (619 mg/l), *E. faecalis* FS 50.1 (616 mg/l), *E. durans* FS 39.1 (570 mg/l), *L. casei* subsp *casei* FS 27.2 (550 mg/l), *L casei* FS 22.2 (523 mg/l), *L. casei* subsp *casei* FS 13.1 (512 mg/l), *E. faecalis* FS 38.2 (512 mg/l), *E. faecium* FS 2.0 (510 mg/l), *L. fermentum* FS 15.2 (509 mg/l) and *L. helveticus* FS 12.6 (506 mg/l) (Table 2). Eighty eight of the isolates produced EPS in the range of 200-500 mg/l while rest of the isolates yielded EPS that was less than 200 mg/l.

Cholesterol-lowering ability of LAB and bifidobacteria isolates

All the LAB and bifidobacteria isolates in the current study expressed cholesterol lowering ability up to a varying extent (Table 2). The isolate *L. plantarum* FS 1.2 showed maximum cholesterol reduction (59.6%) and was followed by isolates *L. plantarum* FS 4.7 (58.09%), *E. faecalis* FS 37.1 (58.02%), *Bifidobacterium* sp. FS 38.3 (57.91%), *L. casei* FS 12.5 (57.65%), *L. helveticus* FS 12.6 (57.02%), *E. faecalis* FS 37.2 (56.20%), *Bifidobacterium* sp. FS 1.3 (54.97%), *L. casei* FS 22.2 (54.42%), *L. casei* subsp. *casei* FS 25.6 (53.36%), *L. plantarum* FS 14.1 (53.20%), *L. helveticus* FS 30.1 (52.94%), *Bifidobacterium* spp. FS 13.3 (52.67%) and *Bifidobacterium* sp. FS 24.1 (52.28%). Thirty five isolates expressed cholesterol reducing ability of 40-52%, while rest of the isolates showed cholesterol reducing ability of 40% or less. Cholesterol at 470 mg/l was used as control.

Antibacterial activity of LAB isolates

Most of the LAB isolates showed antagonistic activity against potential pathogens of human health significance. Only a few isolates showed substantial growth inhibition of the pathogens (inhibitory zone size of 10 mm or above). Five isolates (*Lactobacillus plantarum* 1.2, *Lactobacillus brevis* 28.1, *Enterococcus durans* 23.3, *Enterococcus faecium* 43.2, *Enterococcus faecalis* 50.3) showed maximum inhibition zone (10 mm or above) against *S. aureus*; four isolates (*Lactobacillus helveticus* FS 30.1, *Enterococcus durans* 42.1, *Enterococcus faecalis* 50.2, *Enterococcus faecalis* 31.4) showed against *E. coli;* three isolates (*Lactobacillus plantarum* 5.2, *Lactobacillus casei* subsp *casei* 13.1, *Lactobacillus plantarum* 14.1) showed growth inhibition against *P. aeruginosa;* and three LAB isolates (*Lactobacillus plantarum* 5.2, *Lactobacillus plantarum*

14.1, *Lactobacillus casei* subsp *casei* 13.1) showed antibacterial activity against *Klebsiella* sp., and two isolates (*Lactobacillus plantarum* 14.1, *Lactobacillus plantarum* 29.3) inhibited *E. faecalis*, and two other isolates **(***Enterococcus faecium* 1.1, *Lactobacillus plantarum* 14.4) inhibited *B. subtilis.* Majority of the LAB isolates expressed moderate antagonistic activity against the pathogens examined (inhibition zone 5-10 mm) while others exhibited weak inhibitory activity (inhibition zone size 5 mm or less). Only one isolate (*L. plantarum* FS-11.2) showed maximum inhibition against *P. alcaligenes* whilst none of the isolate expressed maximum inhibition against *B. cereus*. The isolate earmarked as *E. faecalis* FS1.1 and *L. plantarum* FS 1.2 were most distinguished as they not only inhibited a majority of pathogens but also showed the maximum zone of inhibition.

Earmarked most potential probiotic isolates

Based upon the detailed investigation of desired probiotics attributes, three most promising LAB isolates which exhibited most of the features desirable for probiotics viz. *Enterococcus faecium* FS 1.1 (Fig. 1a), *E. faecium* FS 2.0 (Fig. 1b), and *E. faecium* FS 4.0 (Fig. 1c), were earmarked and identified based on 16S rDNA sequence analysis (Fig. 1).

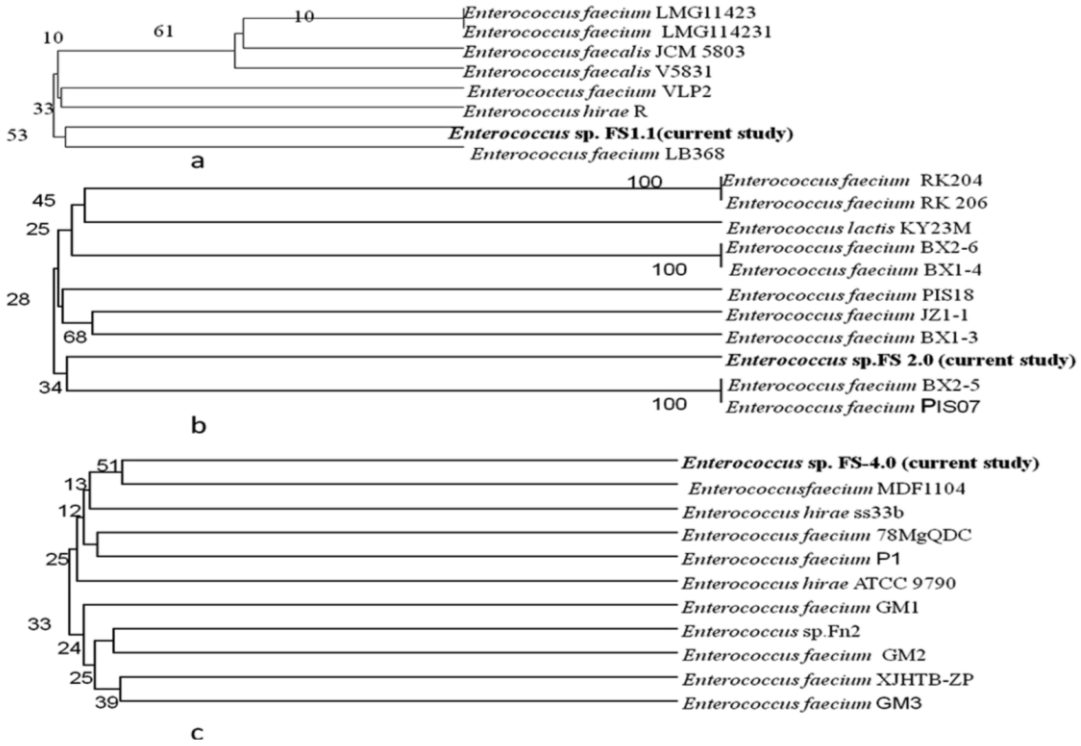

Figure 1 Identification of most promising LAB isolates *Enterococcus faecium* FS 1.1 (a), *E. faecium* FS 2.0 (b) and *E. faecium* FS 4.0 (c) by 16S rDNA sequence analysis. Dendrogram were generated by using p-difference model and UPGMA statistical approach

Fecal enzyme analysis (β-glucuronidase and β-glucosidase)

β-glucuronidase activity was higher in the formula fed infants as compared to the breast fed ones. The highest average activity of β-glucuronidase (0.76 mmoles/mg protein) was observed in formula fed infants (4-6 months) while the lowest activity (0.23 mmoles/mg protein) was observed in breast fed infants in the age band of 0-2 months (Fig. 2a). β-glucosidase activity analysis in breast fed and formula fed infants upto six months of age showed that breast fed infants had higher levels of β-glucosidase as compared to formula fed ones. The maximum β-glucosidase activity (0.7 mmoles/mg protein) was reported from breast fed infants in the group of 4-6 months while the formula fed infants in 0-2 months of age exhibited lowest β-glucosidase activity (0.23 mmoles/mg protein) as presented in Fig. 2b). Furthermore, it was observed that activity of both the enzymes increase with increase in age of infant (Fig. 2c).

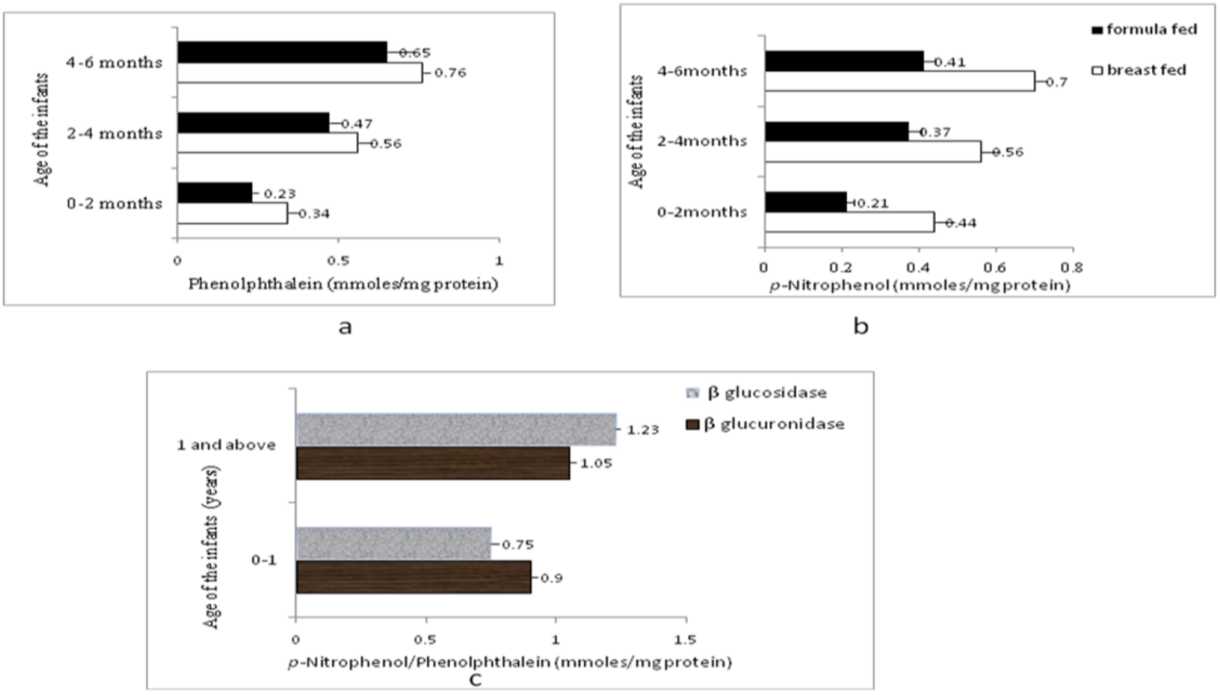

Figure 2 Fecal analysis for activity of β-glucuronidase (a) and β-glucosidase (b), activity variation of these enzymes with infant age (c)

DISCUSSION

The neonatal intestinal ecosystem is quite complex and performs variety of nutritive, immunological and protective functions (**Turroni *et al*., 2012; Ozen and Dinleyici, 2015**)). Generally *Bifidobacterium* spp. dominates in the gut of an infant borne out of vaginal mode of delivery and exclusively breast-fed; relatively fewer enterobacteria and enterococci may be present but obligate anaerobes like *Clostridium* and *Bacteroides* spp. are quite rare (**Fanaro *et al*., 2003; Langhendries 2005**). However, the current study reports a higher number of lactobacilli and cocci as compared to the *Bifidobacterium* spp. (Table 1). This is contrary to the widely published reports that *Bifidobacterium* spp. constitutes the most abundant component of infant gut microflora (**Turroni *et al*., 2012**). However, there are some inconsistencies in literature regarding the prevalence, diversity and abundance of *Bifidobacterium* spp. in the infant intestinal microbiota. Initial bacterial colonization of the infant gut is strongly dependent on maternal flora, conditions and environment during delivery, and to certain extent nutritional habits (**Fanaro *et al*., 2003**). In addition, social, cultural and economic factors may have significant influence on infant initial gut colonization. Furthermore, intestinal microflora of infants born in rural, suburban and urban areas, and babies born at home and at hospitals do differ considerably (**Fanaro *et al*., 2003**). Variation in hygiene during delivery or during breast feeding, and antibiotics usage may be the probable reasons for differences in infants' initial microflora. In the Indian sub-continent particularly in the rural setup, there are rituals according to which the new born are given their first ever feed which may include honey, jaggery, glucose-water, plane water, top milk and gripe water etc. which may have considerable influence on initial gut microflora composition.

Inherent variations among probiotic strains with respect to their health benefitting attributes have been the motivation to investigate and target novel efficacious strains **(Ozen and Dinleyici, 2015; Bajaj *et al*., 2015**). Most important prerequisite for probiotics is to survive in the human gastrointestinal tract, and the ability to withstand low pH, bile, salt and phenol (**Bajaj *et al*., 2014**). The probiotic strains investigated in the current study (Table 2) for potential application in the prevention/treatment of pediatric gastrointestinal diseases exhibited tolerance to wide pH range (pH 2-8), suggesting their ability to survive in different parts of the GI tract viz. stomach (pH < 2), small bowel (proximal part pH 4-7, distal part pH 7.8- 9) and large intestine (pH 4.0 - 6.0). The pH resistance of probiotics strains has been reported to differ widely and is largely strain dependent **(Bajaj *et al*., 2014; Bajaj *et al*., 2015)**. LAB isolates from dairy products grew well at pH 4 (**Jayakumar *et al*., 2012)** while those isolated from infant feces showed growth over broad pH of 1- 9 (**Pelinescu *et al*., 2011**). Bile salts constitute one of the major factors that influence the viability of LAB in GI tract. Generally, the concentration of bile salts in bile varies from 0.8- 4%. In the current study, all the LAB isolates grew and survived at 2% of bile salt but only fewer thrived at 4% bile (Table 2) representing an advantage for the survival of these bacteria, once introduced in the GI tract, especially at the level of small bowel, where the concentration of bile salts is very high. Infant fecal isolates of LAB showed varying resistance to taurocholic acid sodium salt concentrations at 0.5 - 4% (**Pelinescu *et al*., 2011**).

Resistance to phenol is an important probiotic characteristic as phenol can be formed by deamination of some aromatic amino acids by some bacteria and be able to exert bacteriostatic effect (**Suskovic *et al*., 1997; Bajaj *et al*., 2014**). Most of the LAB strains in this study grew well at 0.2-0.4% phenol (Table 2). *Lactobacillus brevis*, *L. plantarum*, and *Pediococcus* could decarboxylate the phenolic carboxylic acids, ferulic, and p-coumaric acids (**Szwajgier and Jakubczyk, 2010).** The LAB strains from milk products showed phenol tolerance up to 0.4 - 0.6% (**Jayakumar *et al*., 2012**).

Osmotolerance is one of the desired attribute for LAB strains intended for industrial applications (**Adnan and Tan, 2007**). During industrial fermentation using LAB, alkali is pumped to prevent excessive reduction in pH due to lactic acid accumulation, which results in convertion of free acid to salt which in turn increase the osmotic pressure **(Bajaj *et al*., 2014)**. Therefore, LAB strains must have high osmotolerance. All the LAB strains in the current study could hold on well up to 8% of salt levels but a further increase resulted in marked reduction in the growth and survival although some strains were able to do well even at 12% level (Table 2). LAB isolates from dairy sources showed growth and survival at high salt concentration (6%) but growth ceased with further increase in salt concentration up to 8% (**Jayakumar *et al*., 2012**).

Hydrophobicity defines the adherence of bacteria to epithelial cells, and constitute an important probiotic property to prevent adherence and invasion of pathogens (**Martin and Martin, 2009**). Positive correlation between hydrophobicity-adhesion and aggregation-hydrophobicity (**Li *et al*., 2008**) suggests that bacteria with higher hydrophobicity have optimum auto-aggregation as well. Fecal LAB isolates in the present study showed varied level of hydrophobicity (Table 2). Similarly, LAB isolates from milk products showed variable levels of hydrophobicity with different organic solvents (**Jayakumar *et al*., 2012**). Heat treatment has been reported to increase the hydrophobicity (**Collado *et al*., 2007**).

Probiotics have been reported to produce exopolysaccharides (EPS) which may have potential applications in food industries as biothickeners, viscosifying, stabilizing, emulsifying or gelling agents; in addition, EPS have been reported to possess antitumour activity, immunomodulating bioactivity and anticarcinogenicity, thus may have health implications (**Doleyres *et al*., 2005**). In the present study, appreciable EPS producing ability of LAB isolates reflects their potential significance in food and pharmaceutical industries (Table 2). EPS producing ability of various *Lactobacillus* and *Bifidobacterium* strains isolated from the human intestinal microbiota has been investigated (**Madiedo *et al*., 2007**).

High high serum cholesterol level is the major risk factor for coronary heart diseases, according to WHO by 2030 cardiovascular diseases will be the leading cause of death affecting about 23.6 million people around the world (**WHO, 2009**). Several pharmacological agents are available that effectively reduce cholesterol level but these are expensive and have severe side effects **(Bliznakov, 2002**). Probiotics due to their bile salt hydrolase activity may interact with hosts' bile salt metabolism and could reduce cholesterol levels **(De Smet *et al*., 1998; Bajaj *et al*., 2015**). Serum cholesterol is reduced due to its conversion into bile acids. LAB isolates in the current study exhibited potential cholesterol lowering ability (Table 2). Similar to current study, analysis of seven potential probiotics

from human gut showed that *Lactobacillus fermentum* strain KC5b caused maximum cholesterol reduction **(Dora *et al.*, 2003; Bajaj *et al.*, 2015**).
Antimicrobial activity is a desirable probiotic attribute. In the present study, most of the potential probiotic isolates showed a potent inhibitory action against the pathogens of human importance. GI tract infections are one of the major causes of morbidity and mortality among infants/children worldwide; severe diarrhea and dehydration account for 15% of total infants/children deaths per year (**Lopez *et al.*, 2001**). Probiotics may be helpful in preventing and treating some types of bacteria-induced diarrhea because of their ability to alter the activity of the intestinal microflora and compete with potential pathogens (**Marteau *et al.*, 2001; Bajaj *et al.*, 2015**) by the production of acids, hydrogen peroxides or by production of small peptides. The LAB isolates from snake gourd were reported to inhibit the growth of *Shigella sonnei*, *Shigella. Staphylococcus aureus* and *E. coli* (**Awasthy *et al.*, 2008**). Five LAB isolates from dairy products showed antimicrobial potential against *E. coli* and *S. aureus* (**Jayakumar *et al.*, 2012**)
The enzymatic activities of gut microbiota has been identified as one of the indicator for global assessment of gut microbiota functions in the host, and has been reported to be associated with human health and disease status (**Humblot et al., 2007**). Activity of β-glucuronidase has been identified as a conserved function among bacteria colonizing the human gastrointestinal tract, which by uncoupling glucuronides, can deconjugate potential toxins increasing the formation of carcinogens in the bowel and promoting the enterohepatic recirculation of toxins, hormones and various drugs (**Adlercreutz *et al.*, 1976; Kuhn, 1998**) in the body. Elevated levels of β-glucuronidase may be a primary factor in the etiology of colon cancer (**Kuhn, 1998; Humblot et al., 2007**), increased risk of breast cancer in postmenopausal women who have high estrogen levels (**Key *et al.*, 2011**). In the current study, LAB isolates from the breast fed infants showed lower levels of β-glucuronidase which reflects the importance of breast feeding towards the development of a general well being of the infant (all LAB were from breast fed infants). β-glucosidases seems to have a more general role in the bioavailability of plant polyphenols and the extraction of energy from insoluble fibers and other indigestible carbohydrates **(Han *et al.*, 2007**). The breast fed infants showed higher level of β-glucosidase as compared to the formula fed infants. The present study in general portrays variation in fecal enzyme levels of infants with respect to mode of delivery, diet and age (**Mykkanen *et al.*, 1998)** also attributed a change in age and adoption of an adult type diet towards the variation in fecal enzyme levels. Enzymes in LAB isolates from human feces were studied and it was reported that the activity of β-glucuronidase increased with age while that of β -glucosidase decreased **(Mroczynska and Libudzisz, 2010**)

CONCLUSION

Current study concludes that *Lactobacillus* spp. constitute the most predominant spp. (*L. plantarum* being the most abundant) contrary to the previous reports which claim *Bifidobacterium* spp. to be the predominant. Several of the LAB isolates from infant gut exhibited tolerance to pH, NaCl, bile and phenol concentration indicating their ability to thrive in the gut environment. Some of the LAB isolates possessed excellent probiotic features such as high hydrophobicity, cholesterol lowering ability, EPS producing ability and antagonistic activity against pathogens, but three isolates (*Enterococcus faecium* FS 1.1, *E. faecium* FS 2.0, *E. faecium* FS 4.0) showed most promising potential as probiotics. Breast fed infants showed lower level of β-glucuronidase, and higher level of β–glucosidase as compared to formula fed ones which underlines the health benefits of breast feeding for infants. Further in depth studies must be conducted to assess the potential of LAB isolates for perspective application as infant probiotics.

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