




Isomaltooligosaccharides utilization and genomic characterization of human infant anti-inflammatory *Bifidobacterium longum* and *Bifidobacterium breve* strains

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Abstract

This study was carried out to understand the probiotic features, ability to utilize non-digestible carbohydrates and comparative genomics of anti-inflammatory *Bifidobacterium* strains isolated from human infant stool samples. Bacterial strains were isolated from the stool samples using serial dilution on MRS agar plates supplemented with 0.05% L-cysteine hydrochloride and mupirocin. Molecular characterization of the strains was carried out by 16S rRNA gene sequencing. Anti-inflammatory activity was determined using TNF- α and lipopolysaccharide (LPS) induced inflammation in Caco2 cells. Probiotic attributes were determined as per the established protocols. Isomaltooligosaccharides (IMOS) utilization was determined in the broth cultures. Whole genome sequencing and analysis was carried out for three strains. Four obligate anaerobic, Gram positive *Bifidobacterium* strains were isolated from the infant stool samples. Strains were identified as *Bifidobacterium longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16. The strains were able to prevent inflammation in the Caco2 cells through lowering of IL8 production that was caused by TNF- α and LPS treatment. The strains exhibited desirable probiotic attributes such as acid and bile tolerance, mucin binding, antimicrobial activity, bile salt hydrolase activity, cholesterol lowering ability and could ferment non-digestible carbohydrates such as isomaltooligosaccharides and raffinose. Furthermore, Isomaltooligosaccharides supported the optimum growth of the strains in vitro, which was comparable to that on glucose. Strains could metabolize IMOS through cell associated α -glucosidase activity. Genomic features revealed the presence of genes responsible for the utilization of IMOS and for the probiotic attributes.

Keywords Anti-inflammatory activity · *Bifidobacterium* · Mucin binding · Prebiotics · Short chain fatty acids

Introduction

Shifting eating habits from traditional to the westernized diets and irrational use of antibiotics causes dysbiosis in the gut microbiota and associated diseases, such as obesity,

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insulin resistance, type 2 diabetes, cancer, mental illnesses, acute and chronic inflammatory conditions, such as ulcerative colitis, irritable bowel disease (IBD) (Woodmansey 2007; Valdes et al. 2018). Foods that are rich in dietary fibers, probiotics, prebiotics and their combination as synbiotics help in the restoration or replenishment of the microbiota and decreasing the disease burden (Kondepudi et al. 2012; Gagliardi et al. 2018). Probiotics are the live microorganisms and when administered in an adequate amount has been suggested to improve the host's health. Among the widely studied probiotic bacteria, *Bifidobacterium* genus inhabit the gut of humans and animals with higher abundance of *B. longum*, *B. breve*, and *B. bifidum* during human infancy, while ageing population has higher abundance of *B. catenulatum*, *B. adolescentis* and *B. longum* spp due to high intake of plant-derived carbohydrates (Arboleya et al. 2016). *Bifidobacterium* is largely known to boost the innate and adaptive immunity (Ruiz et al. 2017), suppress pathogen invasion by competitive exclusion (Bermudez-Brito et al. 2012), production of antimicrobial peptides (Cazorla et al. 2018) and short chain fatty acids (Usta-Gorgun and Yilmaz-Ersan 2020), besides utilizing non-digestible carbohydrate polymers and oligomers (Fu et al. 2019). Studies on animals and humans suggested the protective efficacy of some of the probiotic strains against enteric infections, antibiotic associated diarrhea (AAD), intestinal bowel disease IBD, ulcerative colitis and colorectal cancer studies (Kajander et al. 2008). Recent studies also suggest that *Bifidobacterium* strains could promote metabolic and mental health by preventing the loss of intestinal barrier function and suppressing gut inflammation (Sarkar et al. 2016). Some of the studies on Indian origin *Bifidobacterium* strains includes that on acid resistant and immuno-modulatory *Bifidobacterium breve* NCIM 5671, *Bifidobacterium longum* NCIM 5672 and *Bifidobacterium bifidum* NCIM 5697 isolated from Indian infants (Achi et al. 2019; Rohith and Halami, 2021; Sundararaman et al. 2021) and a *Bifidobaacterium* enriched ice cream (Kataria et al. 2018).

Prebiotics are non-digestible substances, especially oligosaccharides, which are metabolized by the gut microbes and modulate the composition and/or activity of the gut microbiome, thus conferring a beneficial physiological effect on the host (Guarino et al. 2020). International Society for Probiotics and Prebiotics recommended a consensus definition for prebiotics as a substrate which is selectively utilized by the host microorganisms (Gibson et al. 2017). Gut microbial action on prebiotics generate acetate, butyrate and propionate, the key secondary metabolites, in the gut and those can help in maintaining gut-barrier homeostasis, mucus production, gut hormone secretion, systemic beneficial effects, improving calcium and magnesium availability and majorly inhibition of enteric pathogens (Turroni et al. 2016). Several studies suggested that the prebiotics promote

the *bifidobacterial* growth (bifidogenic effect) in the gut (Meyer and Stasse-Wolthuis 2009; Kumar et al. 2020). As there are limited studies on the utility of *Bifidobacterium* strains isolated from Indian infants for their health promoting properties, the present study was aimed at the isolation of *Bifidobacterium* strains that could prevent TNF- α and LPS induced intestinal epithelial cell inflammation using in vitro studies, their probiotic characterization and prebiotic preferences.

Materials and methods

Materials

MRS broth, MRS agar, phenol, lactic acid (HPLC grade) and other chemicals were purchased from HiMedia Laboratories, Mumbai, India. The DNA isolation kit was purchased from ZR Zymo (Zymo Research Corporation, CA, USA). Mucin type III from porcine stomach, fructooligosaccharides (FOS), inulin from chicory, carboxyfluorescein diacetate (CFDA), 2-Nitrophenyl α -D-galactopyranoside (PNP- α -D-galactose), 2-Nitrophenyl β -D-galactopyranoside (PNP- β -D-galactose) and volatile Short chain fatty acid Mix (HPLC) were purchased from Sigma-Aldrich, St Louis, USA. Isomaltooligosaccharides (IMOS) was a gift from BioNeutra, Alberta, Canada. Xylene, chloroform, hexane, and hydrochloric acid were purchased from Merck, Kenilworth, USA. API CHL 50 kit purchased from BioMérieux, Craponne, France. Ethanol estimation kit was purchased from Megazyme International Ireland Limited (Pro Lab Marketing PVT. LTD., New Delhi, India). Agilent Hi-Plex columns (Zorbax Hi-Plex H, 300 \times 7.7 mm HPLC column; 8 μ m particle size and Hi-Plex H cartridge, 3 \times 5 mm internal diameter) were purchased from Agilent technologies, Santa Clara, USA. Caco-2 cell line was purchased from the National Centre for Cell Science, Pune, India. Anoxomat and anoxomat jars used for creating anaerobic conditions were purchased from Mart Microbiology (Model no: AN2CTS), Drachten, The Netherlands.

Methods

Isolation and identification of *Bifidobacterium* strains

Fecal samples were collected from Indian infants of the Tricity region (2–4 months, born by natural delivery) as per the laws and guidelines of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and after the institute's ethical committee approval (No. P-641) and informed consent from the parents. Fecal samples were thoroughly homogenized and were serially diluted on MRS agar plates supplemented with 0.05% (w/v) each of L-Cysteine

hydrochloride and mupirocin. Plates were incubated under anaerobic conditions (80% N₂, 10% CO₂ and 10% H₂) in an anaeromat jar at 37 °C for 48 h. A single colony of the strains was streaked on the fresh MRSC agar plates to obtain the pure cultures and the isolated strains were subcultured and stored as glycerol stock cultures at – 80 °C. A loop full of glycerol stock of the strains was activated on MRSC plates and incubated at 37 °C for 48 h under anaerobic conditions. A single colony of the strain was inoculated into fresh pre-reduced MRSC broth and incubated at 37 °C for 24 h and transferred three times. The cultures were centrifuged at 9000×g for 10 min at 4 °C and the pellets obtained were washed and suspended in sterile phosphate buffer saline with 0.05% L-cysteine (PBSC) and used in all the experiments.

Molecular characterization: Strains were grown in the MRSC broth till logarithmic growth and the genomic DNA was extracted using ZR Zymo DNA isolation kit as per manufacturer's instructions. The presence of *fructose 6-phosphate phosphoketolase* gene was determined using PCR amplification using XFPF 5'-CGGCCACGGCTGGGCC-3' and XFPR 5'-TCCTGACGCCAGACGTGGG-3' primers. PCR amplification of the *16S rRNA gene* was carried out using universal primers 27F 5'-AGAGTTTGATCTGGCTGAG 3', 1492R-5' GGTTACCTTGTTACGACTT 3' primers. The amplicons were sequenced and the sequences were subjected to nucleotide blast using the NCBI database server. Accession numbers for the 16S rRNA gene sequences for *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 were MK850096, MK850097, MK850098 and MK910584, respectively, and are available in the GenBank. *B. longum* Bif10, *B. breve* Bif11 and *B. longum* Bif16 were deposited under Budapest treaty at Microbial Type Culture Collection (MTCC), CSIR-IMTECH, Chandigarh with MTCC 25245, MTCC 25246 and MTCC 25247 as the accession numbers (Supplementary Table 1).

Biochemical tests Catalase, Haemolytic pattern and antibiotic susceptibility were determined as per the standard biochemical assays for bacteria. Degradation of gastric mucin by the *Bifidobacterium* strains was evaluated using an agar plate method developed by Zhou et al. (2001). Carbohydrate fermentation pattern of the strains was determined using API CHL 50 kit as per the manufacturer's instructions and the change in colour of the medium was recorded.

In vitro protective effect on human intestinal epithelial cell (Caco2) inflammation: Caco2 cells were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C under 5% CO₂ in a humidified incubator. Media was changed on every second day until confluency was attained (15 days). Caco2 cells were treated with the four *Bifidobacterium* strains for 24 h and the viability was determined using the MTT assay kit as per the manufacturer's instructions. The ability of the strains to

prevent the TNF-α and LPS-induced inflammation in the Caco2 cells was determined as described by Singh et al. (2018). Briefly, the confluent Caco2 cells were treated with TNF-α (100 ng/mL) for 24 h followed by LPS alone (to induce inflammation) or with a combination of LPS and test strains of OD_{600nm} of 1.0 (OD_{600nm} 1.0 corresponds to 10¹⁰ CFU as determined by plate count method) in DMEM for another 24 h. Later, the supernatants were collected from respective treatments and the level of IL-8 was determined using commercially available IL-8 measurement kit as per the manufacturer's instructions.

In-vitro probiotic characterization:

Acid and bile tolerance, anti-microbial activity, cholesterol lowering activity, bile salt hydrolase (BSH) activity, adhesion to mucin, auto-aggregation and salt aggregation were determined as described elsewhere (Singh et al. 2018). Detailed description is given in Supplementary methods.

Prebiotic Profiling

This was determined for newly isolated strains by agar plate and batch fermentation assays. *B. breve* Bif40 isolated from VSL#3 was used as a reference strain for *B. breve* Bif11 and *B. longum* JCM 1217 procured from Riken Culture Collection, Japan was used as reference strain for *B. longum* Bif10, *B. longum* Bif12 and *B. longum* Bif16.

Agar plate assay Single colony of the strains was spotted on 0.5% (v/v) prebiotic–MRSC plates (pH 6.8) supplemented either with IMOS, FOS, inulin, starch or resistant starch (RS). Color change from red to yellow of phenol red as pH indicator was recorded and a score was given from 1 to 3 depending upon width (mm) of the zone around the spot.

Batch Fermentation in IMOS and raffinose containing medium Bacterial strains were inoculated into MRSC-basal broth (MRSC-BB) supplemented with either 1% of IMOS or raffinose as a prebiotic source. Growth of the strains in MRSC-BB supplemented with 1% glucose was used as a positive control, while growth in MRSC-BB without any carbohydrate source was used as a negative control.

Growth profiles in IMOS The bacterial cells were adjusted to an initial OD_{600nm} of 0.05 units in 5 mL of MRSC-BB supplemented with 1% each of glucose, IMOS and raffinose separately and were incubated under anaerobic conditions in anaeromat jars at 37 °C. Growth was determined by measuring absorbance at OD_{600nm} for every 12 h interval up to 72 h. Doubling time in each carbohydrate substrate was determined in 96 well plates by measuring OD_{600nm} for every 4 h up to 16 h.

Doubling time was calculated using following formula:

$$\text{Doubling time} = \ln(2)/r$$

where r is the growth rate which is calculated using formula:

$$r = (\ln\{\text{OD}_2/\text{OD}_1\})/(\text{T}_2 - \text{T}_1).$$

Change in pH was determined in the broth after 72 h of incubation for each strain. The relative bacterial growth (%) for each strain in IMOS and raffinose was determined by comparing the $\text{OD}_{600\text{nm}}$ values of glucose grown cells, which was considered as 100%.

Biomass (g/L) of the strains obtained in MRSC broth supplemented with 1% either of glucose or IMOS was determined by subtracting the initial dry weight of washed cells at time zero from the dry weight of the cells in the log phase of growth (36 h). Total carbohydrate utilization by the strains in glucose and IMOS was determined using phenol sulphuric acid method (Dubois et al. 1956).

Production of hydrolytic enzymes: Bacterial strains were grown in MRSC-BB supplemented with either 1% of glucose, IMOS or raffinose under anaerobic conditions at 37 °C for 24 h. Bacterial pellets were washed, resuspended in 1 mL of PBSC and the optical density was adjusted to 1.0. Live cells were sonicated (QSonica, LLC, 53 Church Hill RD, Newtown, CT, USA) and centrifuged to obtain the intracellular and cell debris fractions. α -Glucosidase, α -galactosidase and β -galactosidase activities were determined in the whole cells, intracellular extract and cell debris. Briefly, to a mixture of 125 μ l of 2.0 mM PNP- α -D-galactose, PNP- α -D-glucose or PNP- β -D-galactose, 75 μ l autoclave MQ water, a 50 μ l of bacterial suspension or intracellular extracts or cell debris was added and incubated at 37 °C in a circulating water bath for 30 min. The reaction was stopped by adding 1 mL of 1.0 M sodium carbonate solution and the absorbance was measured at 405 nm. Results obtained were expressed as 1 μ mol of nitrophenol released per minute under the assay conditions.

Production of acetate, lactate and ethanol Acetate and lactate production by the *Bifidobacterium* strains were determined using high performance liquid chromatography (HPLC) as described by Singh et al. (2016). Full methodology is given in Supplementary methods. Ethanol production was estimated using commercially available ethanol estimation kit as per the manufacturer's instructions.

Effect of growth in IMOS on acid and bile tolerance, adhesion to mucin and cell surface hydrophobicity:

Bifidobacterium strains were grown in 1% either of glucose or IMOS. Acid and bile tolerance, mucin adhesion and cell surface hydrophobicity were determined as described by Singh et al. (2018). Full methodology is given in Supplementary methods.

Genome sequencing, assembly, and gene annotations and comparative genomic analysis:

As the strain *B. longum* Bif12 and *B. longum* Bif16 were isolated from the same fecal sample, strains *B. longum* Bif10, *B. breve* 11 and *B. longum* Bif16 were short listed for whole genome sequencing and for *in-silico* studies. The genomic DNA isolated from these strains was sequenced using the Illumina-HiSeq 1000 technology and the genome assembly was performed in CLC Genomics Workbench (Chander et al. 2020). The genome annotations for *B. longum* Bif10, *B. breve* Bif11 and *B. longum* Bif16 were performed using Rapid Annotation Server and Technology (RAST) (Aziz et al. 2008). Comparative genomics to evaluate presence or absence of a particular gene and *in silico* search of candidate genes responsible for probiotic traits was carried out in RAST annotated genomes and was further confirmed by BLASTn to analyze genes for probiotic properties. BLASTp was used to predict the presence of specific genes or their homologues in respective genome sequences available at the genome database of NCBI. CRISPRCas-Finder was used to predict CRISPR sequences in the genome sequences (Couvin et al. 2018). Furthermore, antiSMASH tool version 2.0 (Blin et al. 2019), bacteriocin genome mining tool and BAGEL version 3 (Van Heel et al. 2018) web tools were used for identifying the gene clusters for secondary metabolites, bacteriocin, or lantibiotics. Locus IDs of IMOS utilization by *Bifidobacterium* genes were deduced from their whole genomes and prediction of genes were based on CAZymes pipeline and BLASTp of the genes available in public domain. The SWISS-MODEL was used for predicting the function of each gene present in the locus. The signalP-5.0 server was used for predicting the signal peptide in each gene in the locus. The assembly and draft genome sequence of *B. longum* Bif10, *B. breve* Bif11 and *B. longum* Bif16 are available as a whole-genome shotgun (WGS) project in GenBank under accession numbers QZM00000000, QELD00000000 and QCZN00000000.

Statistical analysis

PRISM-8.0 software was used (GraphPad software Inc., CA, USA) for all the statistical analyses. All the values were expressed as Mean \pm SEM.

For the experiments on Caco2 cell viability and IL-8 production, cholesterol reduction, antimicrobial activity and relative bacterial growth, the data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. For mucin binding assays, doubling time, biomass, acetate, lactate and ethanol production, the data were analyzed using t test. For acid and bile tolerance in presence of IMOS and Glucose grown cells, the data were analyzed using two-way ANOVA followed by Tukey's Multiple comparisons test. In all the experiments $P \leq 0.05$ was considered as significant.

Results

Isolation and molecular characterization of *Bifidobacterium* strains

Four bacterial strains designated as Bif10, Bif11, Bif12 and Bif16 were isolated from the feces of healthy human infants. The strains were pale white, circular, convex, glistening and opaque colonies with entire margin. Bif10 and Bif11 colonies were non-mucilaginous and powdery in texture, while Bif12 and Bif16 colonies were mucilaginous and sticky in texture. All strains were anaerobic, Gram positive and showed Y- to pleomorphic shapes under the microscope (40× and 100× oil emulsion). Biochemical analysis indicated that the strains were catalase negative, γ -haemolytic and do not ferment mucin, when grown on mucin agar or in mucin broth. The presence of *fructose 6-phosphate phosphoketolase* gene confirmed the strains were *Bifidobacterium* spp. The 16S rRNA gene sequencing and blast analysis of Bif10, Bif11, Bif12 and Bif16 showed 99% homology with *B. longum*, *B. breve*, *B. longum* and *B. longum*, respectively, and they were named as *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16, respectively (Supplementary Table 1).

Effect on intestinal epithelial cell inflammation: Human intestinal epithelial cells (Caco2 cells) showed 100% viability when treated with *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 (Fig. 1A). Furthermore, the four strains curtailed the TNF- α and LPS

induced inflammation in the human intestinal epithelial cells as there was a reduction in IL-8 production which otherwise stimulated by TNF- α and LPS in the colonic cells (Fig. 1B).

Biochemical characterization

Antibiotics susceptibility The effect of antibiotic treatment on the four strains is given in the Supplementary Table 2. *B. longum* Bif10 was sensitive to all the antibiotics except sulphatriad; *B. breve* Bif11 was sensitive towards all the antibiotics except lincomycin, ciprofloxacin, co-trimoxazol and sulphatriad; and *B. longum* Bif12 was sensitive towards all antibiotics except cloxacillin, ciprofloxacin, co-trimoxazol and levofloxacin. *B. longum* Bif 16 was sensitive towards all antibiotics except ciprofloxacin, co-trimoxazole and sulphatriad.

Fermentation towards different carbohydrates Strain variations in the utilization of various carbohydrates was evident among *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 in API-CHL 50 assay (Supplementary Table 3). *B. longum* Bif10 fermented 15 carbohydrates, *B. breve* Bif11 fermented 22 carbohydrates, *B. longum* Bif12 and *B. longum* Bif16 fermented 16 carbohydrates (Supplementary Table 3).

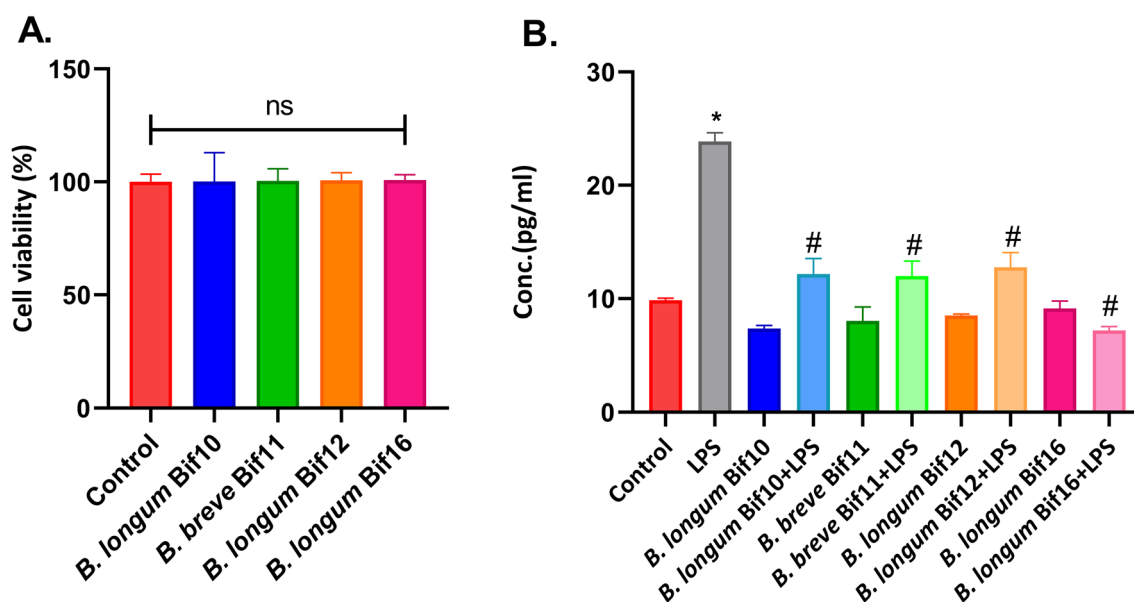


Fig. 1 Effect of *Bifidobacterium* strains on **A** viability of Caco2 cells and **B** IL-8 production by the TNF- α and LPS stimulated Caco2 cells. Note: Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ vs control; # $P < 0.05$ vs LPS

Probiotic attributes

Acid and bile tolerance In acidic growth conditions, *B. longum* Bif10 showed 50, 82 and 84% survivability at pH 2.5, 3.0, and 3.5. *B. breve* Bif11 showed 57, 90 and 92% survivability at pH 2.5, 3.0, and 3.5. *B. longum* Bif12 showed 55, 74 and 76% survivability at pH 2.5, 3.0, and 3.5. *B. longum* Bif16 showed 62, 81 and 91% survivability at pH 2.5, 3.0, and 3.5, respectively (Table 1).

In presence of 0.2 and 0.4% bile, the viability of *B. longum* Bif10 was 81 and 87%. Viability of *B. breve* Bif11 was 92 and 93%, *B. longum* Bif12 was 82 and 84% and *B. longum* Bif16 was 80 and 89% viability in 0.2% bile and 0.4%, respectively (Table 1).

Adhesion to gastric mucin: There was a strain dependent binding to porcine gastric mucin. *B. longum* Bif10, *B. longum* Bif12 and *B. longum* Bif16 showed 10, 25 and 13%

each of mucin binding, whereas *B. breve* Bif11 showed maximum of 56% mucin binding (Fig. 2A). None of the strains showed mucin degradation on mucin agar plates or liquid broth.

Cell surface properties

Auto-aggregation Ability of the strains to auto-aggregate is given in Supplementary Table 4. *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 showed 41, 7, 22, and 5% auto-aggregation, respectively.

Cell surface hydrophobicity: *B. longum* Bif10 showed 61, 55 and 58%, *B. breve* Bif11 showed 16, 17 and 14%, *B. longum* Bif12 showed 40, 28 and 56% and *B. longum* Bif16 showed 10, 8 and 12% hydrophobicity in xylene, hexane, and chloroform, respectively (Supplementary Table 4).

Table 1 Effect of IMOS and glucose on acid and bile tolerance of *Bifidobacterium* strains

Bacterial strain	Acid Tolerance (log CFU/ml)						Bile Tolerance (log CFU/ml)			
	pH 2.5		pH 3		pH 3.5		0.2% Bile		0.4% Bile	
	IMOS	Glucose	IMOS	Glucose	IMOS	Glucose	IMOS	Glucose	IMOS	Glucose
<i>B. longum</i> Bif10	58.3±3	50.0±4	86.3±2*	82.2±2 [#]	90.1±4	84.8±2	86.4±3	81.2±1	88.4±2	86.9±3
<i>B. breve</i> Bif11	67.0±2	56.8±3	95.6±1*	90.0±1 [#]	96.4±1	92.3±1	93.2±2	91.8±2	94.4±12	92.9±1
<i>B. longum</i> Bif12	56.2±6	54.6±2	79.4±1*	74.3±3 [#]	82.1±1	75.9±2	85.7±3	81.8±1	88.1±3	84.0±3
<i>B. longum</i> Bif16	65.6±3	62.5±4	87.5±2*	81.0±2 [#]	92.0±1	90.8±2	88.3±3	80.5±1	92.6±2	89.2±1
<i>B. longum</i> JCM1217	52.1±4	46.7±2	88.4±2*	82.3±1 [#]	89.4±1	84.7±2	82.4±3	80.2±2	85.9±1	81.2±2
<i>B. breve</i> Bif40	48.6±4	38.3±4	82.0±1*	78.0±2 [#]	83.3±2	80.1±3	90.1±1	87.9±1	89.3±3	87.2±3

In acid tolerance assay, comparisons were made for the stains grown in IMOS and glucose and then subjected to acid stress at pHs (2.5, 3.0 and 3.5). In bile tolerance assay, comparisons were made for the stains grown in IMOS and glucose and then subjected to 0.2 and 0.4% of bile stress

Note: Data were analyzed using two-way ANOVA followed by Tukey's Multiple comparisons test

Significance was measured between the respective strains grown in IMOS and glucose at different pH and bile concentrations. Significance * vs IMOS grown cells and [#] vs glucose grown cells

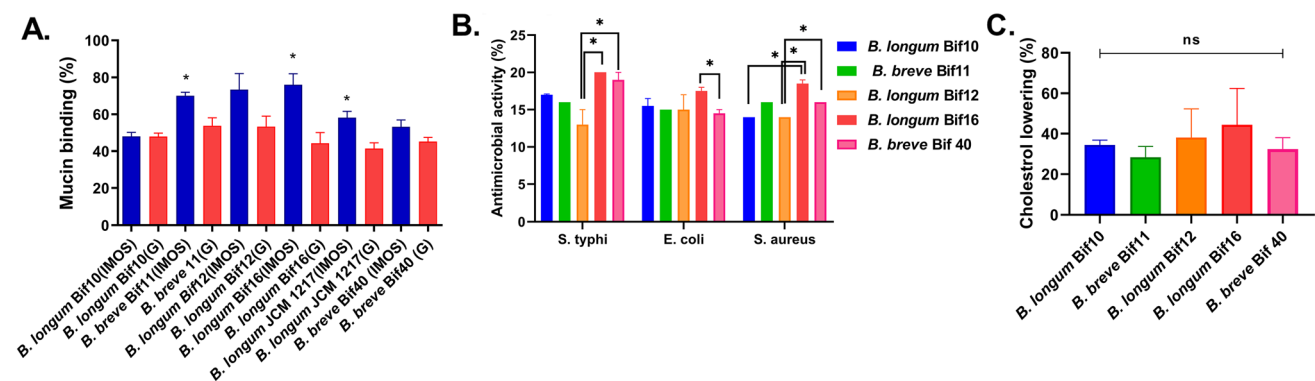


Fig. 2 Probiotic attributes of *Bifidobacterium* strains **A** mucin binding; **B** antimicrobial activity and **C** in vitro cholesterol reduction. Note: Data **A** were analyzed using unpaired *t* test, **B** and **C** were analyzed using one-way ANOVA followed by Tukey's Multiple comparisons test. For mucin binding assay, **P*<0.05 was consid-

ered as significant between each strain grown in MRSC–glucose and MRSC–IMOS, respectively. For antimicrobial activity, **P*<0.05 was considered as significant between the isolated strains and the reference strain *B. breve* Bif40 grown in presence of *S. typhi*, *E. coli* and *S. aureus*, respectively

Congo red binding *Bifidobacterium longum* Bif10 showed 22 and 25%, *B. breve* Bif11 showed 29 and 23%, Bif12 showed 20 and 31% and *B. longum* Bif16 exhibited 17 and 52% of Congo red binding in agar grown and broth grown cells, respectively (Supplementary Table 4).

Salt aggregation test (SAT) *Bifidobacterium longum* Bif10 showed SAT at all molarities (0.2 to 4 M) of ammonium sulphate solution, while *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 showed aggregation at 3.2 and 4 M of ammonium sulphate solution for the agar and broth grown cells (Supplementary Table 4). Interestingly, there was no significant difference in cell surface properties of glucose and IMOS grown cells (Supplementary Table 6).

BSH activity *Bifidobacterium longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 showed positive BSH activity towards TDCA as there was a white precipitate formation surrounding the bacterial colonies as observed in the agar plates. *B. longum* Bif10 showed strong BSH activity as the zone of precipitate formation was 3 mm MRSC–TDCA agar plates, while *B. breve* Bif11 and *B. longum* Bif12 showed moderate activity of 2 mm each. Furthermore, *B. longum* Bif10 also showed a weak BSH activity (1 mm) on MRSC–TCA agar plates, while rest of the strains showed no BSH activity on MRSC–TCA agar plates.

Cholesterol lowering activity *B. longum* Bif16 showed a maximum cholesterol reduction of 45% followed by *B. longum* Bif12 that showed 38% reduction. This was followed by *B. longum* Bif10 which showed 34% and *B. breve* Bif11 showed 28% of cholesterol reduction (Fig. 2C).

Anti-microbial activity Antimicrobial potential of bacterial culture supernatants of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 against *E. coli* (MTCC-3222), *S. aureus* (ATCC-9144) or *S. typhimurium* (NCTC-74) is given in the Fig. 2B. Maximum anti-bacterial activity was exhibited by *B. longum* Bif16 as it showed 20, 18 and 19 mm zone of inhibition against *S. typhimurium*, *E. coli* and *S. aureus*, respectively.

Prebiotic profiling

Agar plate assay suggested that *B. longum* Bif10, *B. breve* Bif11 and *B. longum* Bif16 were able to ferment IMOS. *B. longum* Bif10 alone fermented FOS, while *B. breve* Bif11 alone produced a halo around the colonies on SS and RS agar plates upon staining with iodine solution due to the hydrolysis of SS and RS (Supplementary Table 5). Inulin was not metabolized by any of the strains (Supplementary Table 5).

Growth profiles in IMOS

Bifidobacterium longum Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 showed comparable growth in 1% IMOS in MRSC-BB relative to that in glucose (Fig. 3). The doubling times of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 grown in glucose supplemented broth were 8.9, 8.5, 11.1 and 9.6 h, while in IMOS, the doubling time were 11.0, 10.3, 14.1 and 13.4 h, respectively (Fig. 3I). In the MRSC-BB supplemented with either IMOS or glucose, the pH was reduced to 4.5 from an initial pH of 7.0. Relative bacterial growth of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 in IMOS were 89, 84, 86 and 85%, respectively (Fig. 3E, F). Cell mass of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16 obtained was 2.8, 3.3, 3.0, 3.6, 2.55 and 2.5 g/L, respectively, in MRSC-BB supplemented with IMOS, while on glucose the cell mass obtained were 2.4, 2.6, 3.1, 2.8, 2.36 and 2.11 g/L, respectively (Fig. 3J). In presence of IMOS, the total carbohydrate utilization was 71, 66, 59 and 63% for *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12 and *B. longum* Bif16, respectively, while in glucose, the carbohydrate utilization was 85, 79, 84 and 81% at the end of 72 h of fermentation. *B. longum* JCM 1217 and *B. breve* Bif40 showed 65% utilization of IMOS, while glucose utilization was 78 and 77%.

Hydrolytic enzyme production

α -Glucosidase activity Whole cells of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40 showed 0.19, 0.15, 0.13, 0.13, 0.04 and 0.18 μ M of PNP released per min from respective PNP-glycoside, respectively, when grown on IMOS. Glucose grown culture of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16 and *B. longum* JCM 1217 and *B. breve* Bif40 showed 0.10, 0.11, 0.08, 0.12, 0.05 and 0.15 μ M of PNP formation, respectively (Supplementary Table 7). Intracellular α -glucosidase activity of IMOS grown *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40 was 0.10, 0.12, 0.14, 0.16, 0.10 and 0.18 μ M of PNP release, respectively, while glucose grown cultures showed 0.05, 0.12, 0.10, 0.16, 0.03 and 0.13 μ M of PNP release, respectively (Supplementary Table 7).

α -Galactosidase activity Strains exhibited very less or no extracellular α -galactosidase activity in the culture supernatants. However, whole cells of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40 grown in IMOS showed 0.10, 0.031, 0.035, 0.033, 0.027, and 0.04 μ M of PNP formation, respectively, while the glucose grown cells showed

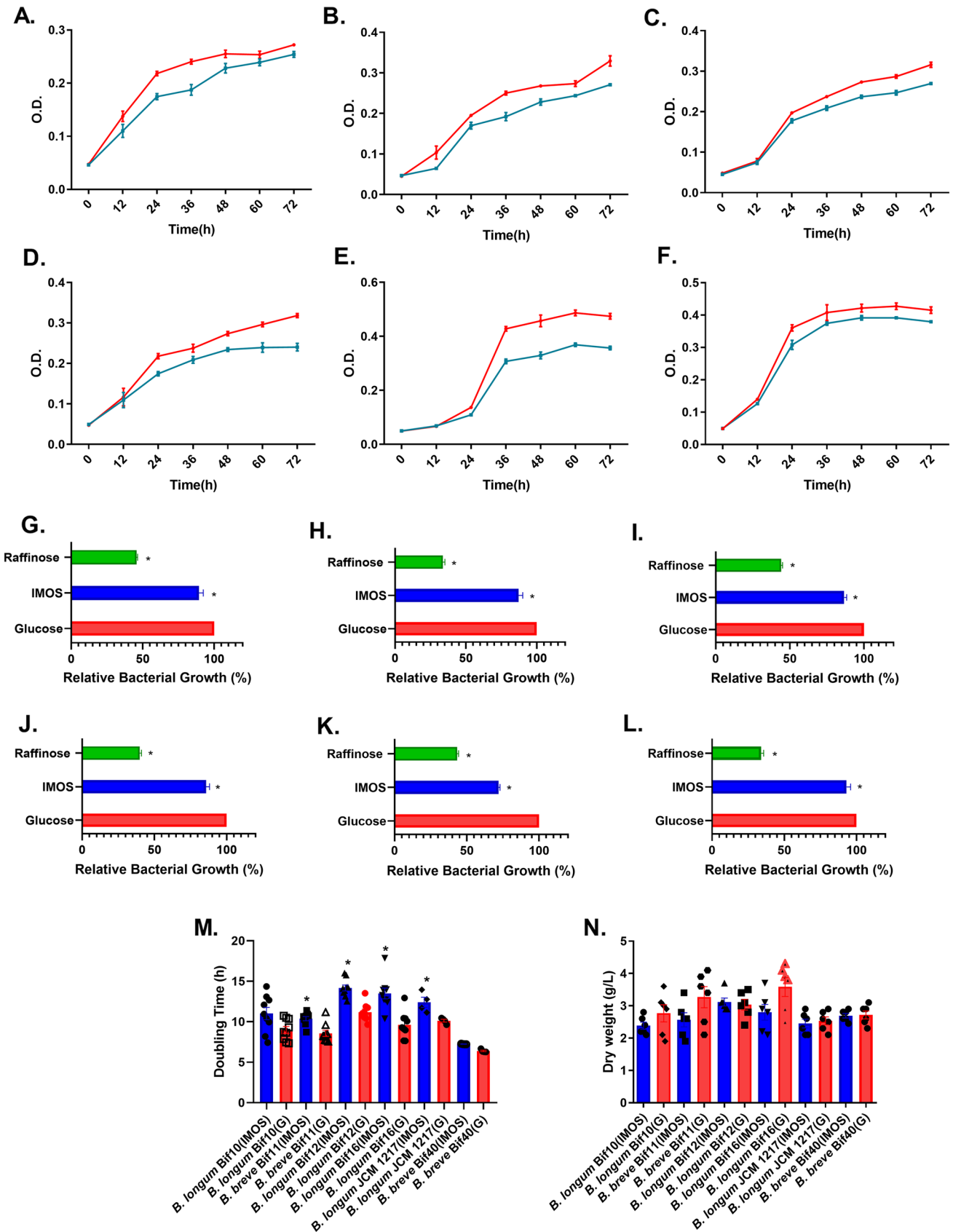


Fig. 3 Effect of IMOS and Raffinose on the growth of *Bifidobacterium* strains. Growth curves (A–F); relative bacterial growth; (G–L), doubling time (M) and dry weight (N) of *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40, respectively. Note: Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ was considered as significance between each strain grown in MRSC–glucose and MRSC–IMOS, respectively. For calculating the relative growth, the growth of strains in MRSC–BB supplemented with glucose was considered as 100%

0.08, 0.03, 0.09, 0.08, 0.02, and 0.03 μM of PNP formation, respectively (Supplementary Table 7).

β -Galactosidase activity Whole cell β -galactosidase activity in *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40 in IMOS was 0.04, 0.05, 0.04, 0.02, 0.06, and 0.05 μM of PNP released, respectively, while the glucose grown cultures showed 0.03, 0.02, 0.04, 0.03, 0.06 and 0.04 μM of PNP production, respectively (Supplementary Table 7).

Production of secondary metabolites

In IMOS broth, acetate production was 24.5, 26.8, 23.4, 24.6, 25.8 and 26.3 mM/mL; lactate production was 14.9, 14.8, 14.32, 14.9, 14.8 and 17.6 mM/mL and ethanol production were 181.3, 180.2, 168.7, 178.4, 206.9 and 177.6 mg/L, respectively, by *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40, respectively. In glucose broth, acetate production was 25.6, 26.6, 26.9, 27.4, 26.8, 24.6 mM/mL; lactate production was 15.7, 15.0, 15.3, 15.2, 16.4 and 17.2 mM/mL and ethanol production was 161.7, 175.5, 190.9, 221.2, 172.3 and 154.8 mg/L, respectively, for *B. longum* Bif10, *B. breve* Bif11, *B. longum* Bif12, *B. longum* Bif16, *B. longum* JCM 1217 and *B. breve* Bif40, respectively (Fig. 4A–C). In case of *B. longum* Bif12 and *B. longum* Bif16 acetate production was higher in IMOS grown cells as compared to that in glucose.

Mucin binding assay *Bifidobacterium longum* Bif10 showed 11 and 30.8%, *B. breve* Bif11 showed 56 and 58%, *B. longum* Bif12 showed 25 and 32.8 and *B. longum* Bif16 showed 13 and 31.4% mucin binding in media supplemented with glucose and IMOS, respectively. *B. breve* Bif40 showed 54.7 and 62% and *B. longum* JCM 1217 showed 48.6 and 54.2% of mucin binding in glucose and IMOS supplemented media, respectively (Fig. 2A).

Genomic features of the *Bifidobacterium* strains

Genome based taxonomy for the three strains using ANI with their type strain reference genome was performed

which confirmed their species status. The general genomic features of all the strains are represented in Table 2. Among the three sequenced genomes, *B. longum* Bif10 had largest genome size (2,631,972 bp), followed by *B. breve* Bif11 (2,494,489) and *B. longum* Bif16 (2,469,902) with typical GC content to their species. Functional classification of genes using RAST subsystem showed the most abundant functional subsystems are amino acids and derivatives, carbohydrates and protein metabolism in all the 3 strains. CRISPRCasFinder had identified 4 CRISPR sequences in *B. longum* Bif10, 6 in *B. breve* Bif11 and 5 in *B. longum* Bif16.

Acid/Gastric response

The genomes of the three strains harbored the genes for *multi antimicrobial extrusion protein Na(+)/drug antiporter*, *MATE family of MDR efflux pumps*, *glyceraldehyde-3-phosphate dehydrogenase*, heat shock protein *DnaK* and chaperones, such as *dnaJ*, *groEL* and *groES* responsible for the acid tolerance (Supplementary Table 8).

Bile/intestinal response

All the three strains showed the presence of *tlyCI* (encoding a hemolysin-like protein), *Choloylglycine hydrolase* (responsible for bile hydrolysis), gene for the B10920 protein, and gene coding for cholate efflux transporter having 97–100% homology with that of *B. longum* BBMN68. The gene for Multidrug resistance protein belongs to the permeases of the major facilitator superfamily and its homologue was present in the three strains, with 62% similarity (296/475). Furthermore, MDR ABC transporters were present in the genomes of the three strains. Thus, 11 such transporters were present in *B. breve* Bif11 and *B. longum* Bif16. Although, strain *B. longum* Bif10 lacks these transporters but it had other unique genes for Multiple sugar ABC transporter, such as *membrane-spanning permease protein MsmG*, *membrane-spanning permease protein MsmF* and *substrate-binding protein* that may perform such functions (Supplementary Table 8).

Genome based metabolic capabilities: carbohydrate utilization

The three strains were able to utilize L-arabinose and D-Ribose that corroborates with genomic features. Ten genes responsible for D-ribose utilization were present in the three strains. Glucose was utilized by the three strains and 9 genes were reported in three strains in the subsystem “Glycolysis and Gluconeogenesis”. Similarly, the three strains harbored fructose-bisphosphate aldolase class II and fructose-6-phosphate phosphoketolase encoding proteins responsible for D-Fructose metabolism (Supplementary

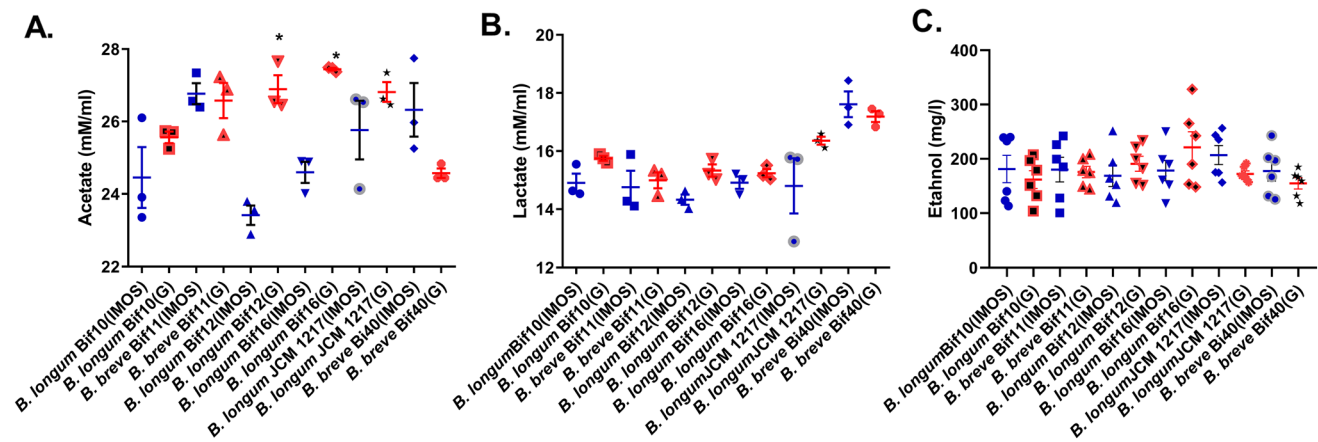


Fig. 4 Effect of IMOS on the production of secondary metabolites **A** acetate; **B** lactate and **C** ethanol. Note: Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ was con-

sidered as significant between each strain grown in MRSC–glucose and MRSC–IMOS separately

Table 2 Genome features of *Bifidobacterium* strains

Parameter	<i>B. longum</i> Bif10	<i>B. breve</i> Bif11	<i>B. longum</i> Bif16
Genome size (bp)	2,617,103	2,472,971	2,447,802
GC content (%)	59.6	59	60
Genes (total)	2406	2231	2205
CDS (total)	2318	2175	2142
Number of subsystems (RAST)	289	277	283
rRNAs	1, 1, 2 (5S, 16S, 23S)	1, 1 (16S, 23S)	1, 1, 3 (5S, 16S, 23S)
tRNAs	80	51	55
ncRNAs	4	3	3
CRISPR Arrays	2	2	2
NCBI accession no	QCZM00000000	QELD00000000	QCZN00000000

Table 9). Batch fermentation study showed that the three strains could utilize raffinose. Gene annotation predicted that all three strains had genes encoding for sucrose phosphorylase and α -galactosidase which cleaves sucrose and melibiose, respectively. Besides this, the genomes of the strains showed various amylases that may help in the utilization of starch and related carbohydrates (Supplementary Table 10).

Genomic determinants for metabolism of maltose and isomaltooligosaccharides All three strains have maltose and maltodextrin utilization locus containing 20 genes. Some of them encode α -glucosidase and associated transporters required for maltose and maltodextrin utilization. Besides these, *B. breve* Bif11 and *B. longum* Bif16 had *maltodextrin glucosidase*, for maltose and maltodextrin utilization (Supplementary Table 11).

Genes for galactose, lactose and oligosaccharides utilization There were six genes that help in the galactose uptake and utilization and 11 genes were involved in D-Lactose

utilization in the subsystem lactose and galactose uptake and utilization (Supplementary Table 12). There were five genes responsible for galactose utilization in strain *B. longum* Bif16. These genes code for α -galactosidase, β -galactosidase, α -galactosidase precursor, evolved β -D-galactosidase (beta subunit) and evolved β -D-galactosidase (alpha subunit). However, *B. longum* Bif10 lacks a gene that encodes for α -galactosidase precursor. *B. breve* Bif11 contains only α -galactosidase and β -galactosidase.

Since all three strains have found to be grown on IMOS, we have nailed down the locus that can encode a set of genes, associating with breakdown of the sugars at extracellular environment and then transport them into cytoplasm, response regulators and a gene for oligo-1,6-glucosidase (GH13_31). Two loci for *B. longum* Bif10 and one each locus for *B. breve* Bif11 and *B. longum* Bif16 were identified. Besides this, multiple oligo-1,6-glucosidase genes were present observed. In case of *B. breve* Bif11 and *B. longum* Bif16, a GH13 hydrolase (α -amylase) degrades large molecules of starch was also observed.

Genomic characterization of Adhesion, Mucin binding and Mucin degradation

Subsystem Sortase Three genes responsible for pilus assemblage and mucin binding were present in the three *Bifidobacterium* strains that encompassed *Sortase A* (LPXTG specific), *cell wall surface anchor family protein* and *NPQTN specific sortase B*.

Within the sialic acid metabolism subsystem, *B. breve* Bif11 contains 15 genes, whereas *B. longum* Bif10 and *B. longum* Bif16 had nine genes each (Supplementary Table 12) responsible for colonization of bacteria in the host gut. Importantly, *B. breve* Bif11 had two important genes, *sialic acid utilization regulator of RpiR family* and *sialidase* that were absent in *B. longum* Bif16 and *B. longum* Bif10. Total four genes responsible for capsular polysaccharides biosynthesis and assembly were present in *B. longum* Bif16, while *B. longum* Bif10 and *B. breve* Bif11 genomes possess only two genes and *lack O-antigen flippase Wzx* and *oligosaccharide repeat unit polymerase Wzy*. In subsystem, rhamnose containing glycans and dTDP-rhamnose synthesis, total nine genes were present in *B. breve* Bif11 and *B. longum* Bif16, while *B. longum* Bif10 had 7 genes (Supplementary Table 12).

Moonlighting proteins

DnaK, *transaldolase*, and *enolase* were present in all the three genomes. However, *EF-Tu* (elongation factor thermo unstable) was present in *B. breve* Bif11 and *B. longum* Bif16 and absent in *B. longum* Bif10.

Secondary metabolites prediction

RAST subsystem predicted the genes for secondary metabolism in *B. longum* Bif16 strain. Furthermore, both antiSMASH and Bagel results predicted the presence of lanthipeptide of putative class II type in *B. longum* Bif16 strain in contig 22 of the assembled genome with complete cluster of size nearly 23 Kb. *B. longum* Bif10 also harbored lanthipeptide cluster of size approximately 20 Kb as predicted by Bagel, which was located in contig 8. The clusters in all the three strains comprised of genes important for immunity, transport & leader cleavage of the predicted lanthipeptide.

Discussion

Majority of the chronic diseases stems due to loss of intestinal barrier function as a result of altered gut microbiota composition and translocation of microbial associated molecular patterns and resultant inflammation (Belkaid and Hand 2014; Singh et al. 2020). In the present study,

a decrease in IL-8 production by the intestinal epithelial cells, an inflammatory marker, suggested that the *Bifidobacterium* strains were able to reduce the inflammation caused due to LPS and TNF- α treatment. Our study is in alignment with an earlier report on reduction in inflammation in LPS stimulated HT-29 cells by *Bifidobacterium* strains through reduction in IL-8 production (Li et al. 2019).

Acid tolerance is the major challenge in case of *Bifidobacteria* and, therefore, is vital for its survival, adaptation to the host and for exerting probiotic functions. In this study, the four *Bifidobacterium* strains showed high resilience towards gastric and bile conditions which are considered as the primary selection criteria for a probiotic strain (Ganguly et al. 2011). Strains in the present study showed 50–90% survivability at a pH of 2.5, 3.0 and 3.5. Genome annotations revealed the existence of *FO/F1-ATPase*, production of ammonia via *cysteine–cystathionine cycle*, *GAPDH*, a moonlighting protein, membrane transporters, such as *major facilitator super family (MFS)* and multiple *antimicrobial extrusion protein family (MATE) transporter families* that helps the bacteria to withstand the acid stress (Corcoran et al. 2005; Antikainen et al. 2007; Lee and O’Sullivan 2010; Andriantsoanirina et al. 2013; Alnaseri et al. 2015; Schindler and Kaatz 2016). Furthermore, the three strains harboured genes for efflux pumps, multidrug transporters, Cholate efflux transporters and bile salt hydrolases that helps the bacteria from bile toxicity and rendering them more tolerant to bile toxicity and making them robust candidates for GIT delivery (Lye et al. 2010). Interestingly, the presence of BSH activity was confirmed using in vitro assays, where the three strains showed positive BSH activity towards TDCA, while *B. longum* Bif10 could degrade TCA.

Besides stress tolerance, adhesion to intestinal epithelial cells and mucus are also the preferred probiotic attributes as suggested by FAO/WHO, 2002. The four strains showed adhesion to epithelial cells and mucin but do not degrade mucin as shown by earlier researchers (Abe et al. 2010). Earlier studies suggested the presence of two and in majority of the cases more types of sortase genes in Gram positive bacteria (Mandlik et al. 2008). Occurrence of *Sortase A* (LPXTG specific), *cell wall surface anchor family protein* and *NPQTN specific sortase B* genes in the three genomes would help in cross-linking individual pilin monomers and thus help in anchoring the bacteria to the intestinal cells (Mandlik et al. 2008). The three strains contained *Wzx* and *Wzy* genes encoding *flippase* that helps in the translocation of O-antigen across the membrane to the cell surface (Islam et al. 2010). Rhamnose-rich O-antigen is responsible for surface attachment, cell–cell aggregation, and biofilm maturation (Islam et al. 2010). *B. breve* Bif11 consists of *sialic acid utilization regulator of RpiR family*, *sialidase* and *LPXTG* that are covalently linked to *sortase A*. This helps in the

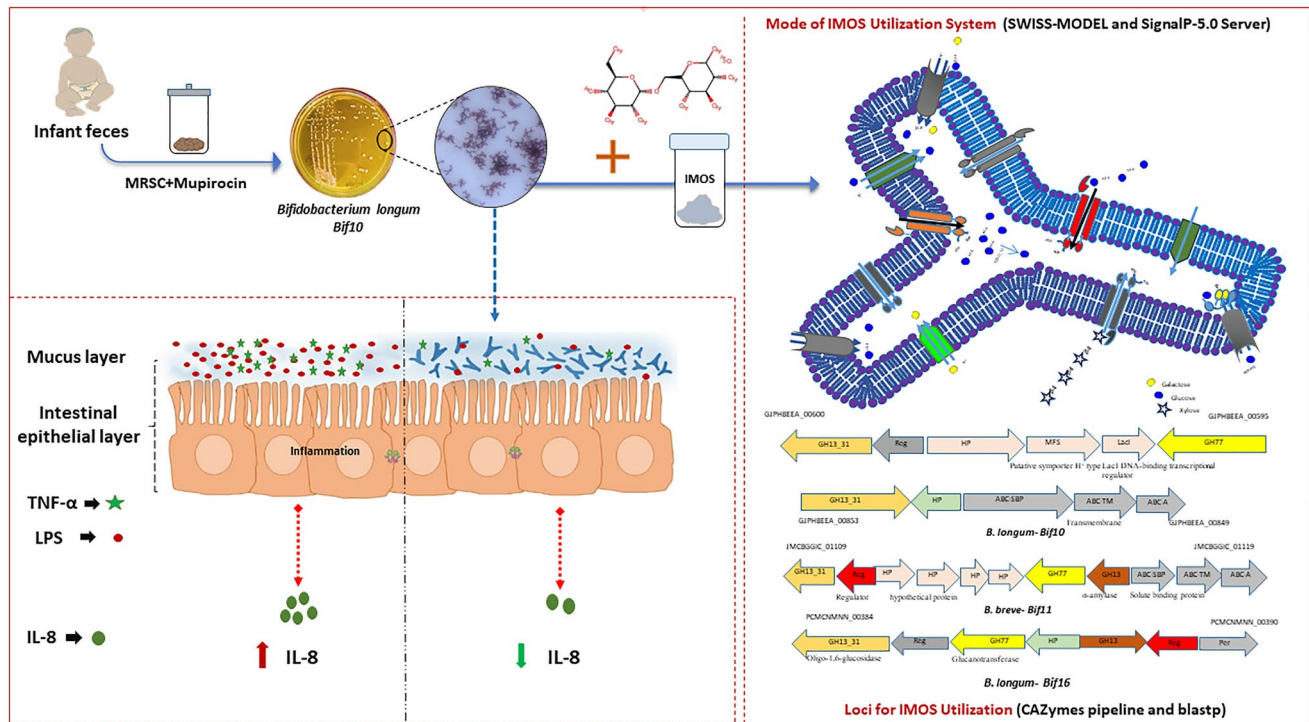


Fig. 5 Summary of the in vitro protective effect of *Bifidobacterium* strains against TNF- α and LPS-induced inflammation in Caco2 cells and the organization of representative loci for utilization of isomaltooligosaccharides (IMOS) in *Bifidobacteria* and mode of IMOS utilization system in the *Bifidobacteria*. Note: Based on sequence

colonization and adhesion of bacteria to cells, such as A549, HeLa and Caco-2 cells.

Antimicrobial activity is one of the desired probiotic features which have been vastly explored by many researchers. In this study, the acid supernatants and not the neutralized ones of the strains inhibit the tested pathogens, which is due to the combined effect of acetic acid, ethanol, lactic acid and H_2O_2 as reported by previous researchers (Marianelli et al. 2010). Interestingly, the genomes of *B. longum* Bif10 and *B. longum* Bif16 contained locus for lanthipeptide and its functional presence and time dependent secretion warrants further studies.

The ability of *Bifidobacterium* strains to utilize/metabolize the dietary and host-derived carbohydrates is highly strain specific and depends on the presence of specific carbohydrases, sugar transporters, such as ABC transporters, which were present in the three strains (Pokusaeva et al. 2011). Growth studies of the strains suggested IMOS as the most suitable non-digestible carbohydrate, as IMOS yielded comparable growth of the strains to that on glucose. Some of the probiotic attributes are improved when the strains were grown with IMOS, although the difference was not significant.

homology from SWISS-MODEL and SignalP-5.0 Server, it has been predicted that IMOS such as isomaltose and isomaltotriose can be taken in the cells by ABC transporter and digested by oligo-1,6-glucosidase in to glucose. Prediction of utilization of other sugars was also made from sequence homology

Genomic studies of the three strains suggested the presence of α -glucosidases, and oligo-1,6 glucosidases required for the breakdown of IMOS, maltose and maltodextrin into their monomeric units. In vitro results augmented the functional presence of cell associated α -glucosidase in the strains when grown on IMOS or glucose in the liquid cultures. Some studies also suggested that saccharides such as lactose, raffinose or IMOS in the fermentation media act as an inducer for α - and β -glucosidases production (Carevic et al. 2016). Since the strains have grown on IMOS, we have identified the locus that can encode for carbohydrases at extracellular environment, and then transport them into cytoplasm, response regulators and a gene for oligo-1,6-glucosidase (GH13_31). Two loci in *B. longum* Bif10 and one locus each in *B. breve* Bif11 and *B. longum* Bif16 was observed, while multiple genes encoding for oligo-1,6-glucosidase were also present. In *B. breve* Bif11 and *B. longum* Bif16, presence of a GH13 (α -amylase) helps in the hydrolysis of polymers such as starch into dextrin forms which are further hydrolysed by GH77 enzymes into oligosaccharides. These oligosaccharides can then be internalized and hydrolysed to monosaccharide units by the action of oligo-1,6-glucosidases and α -1,4-glucosidases in the cytoplasm for further

processing in the energy fermentative pathways. The four strains produce lactate and ethanol besides acetate when grown on IMOS and glucose and hence are heterofermentative. Acetate, through co-feeding can be converted into butyrate by the butyrate producing microbes, a crucial step for achieving the healthy gut homeostasis (Rowland et al. 2018).

Conclusion

Bifidobacterium strains isolated in the present study are metabolically active as they ferment IMOS and galactose-based oligosaccharides and possess desirable probiotic attributes and are anti-inflammatory as they reduce LPS-induced inflammation in the human intestinal epithelial cells (Fig. 5). Hence, these strains could be blended with IMOS or galactose-based oligosaccharides to use as synergistic synbiotics for alleviating inflammatory gut conditions. Detailed studies on the molecular mechanisms for the beneficial effects observed through this study are in progress.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13205-022-03141-2>.

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Author contributions SS¹, SS², KK and MB conceived the research and designed the experiments. SS², KK and SKB isolated the *Bifidobacterium* strains; SS¹, SS², RM and PR conducted the experiments. VC, SM, AC, SR and RS performed the bacterial genomic characterization and in-silico data analysis. SS¹, SS², MB, SKB and KK analyzed the data. SS¹, SS², VC, SM, AC, MB and KK wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest Authors declare no conflict of interest.

Data availability The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Whole genome sequencing data presented in this study are openly available in The National Centre for Biotechnology Information (NCBI).

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Consent for publication Authors undersigned and give the consent for the publication of the given research material in the above journal.

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