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Evaluation of Probiotic Properties and Prebiotic Utilization Potential of *Weissella parameseteroides* Isolated From Fruits

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Abstract

Weissella parameseteroides has gained a considerable attention as bacteriocin and exopolysaccharide producers. However, potential of *W. parameseteroides* to utilize different prebiotics is unexplored area of research. Fruits being vectors of various probiotics, five *W. parameseteroides* strains, namely, FX1, FX2, FX5, FX9, and FX12, were isolated from different fruits. They were screened and selected based on their ability to survive at pH 2.5 and in 1.0% sodium taurocholate, high cell surface hydrophobicity, mucin adhesion, bile-induced biofilm formation, antimicrobial activity (AMA) against selected enteropathogens, and prebiotic utilization ability, implicating the functional properties of these strains. In vitro safety evaluation showed that strains were susceptible to antibiotics except vancomycin and did not harbor any virulent traits such as biogenic amine production, hemolysis, and DNase production. Based on their functionality, two strains FX5 and FX9 were selected for prebiotic utilization studies by thin layer chromatography (TLC) and short-chain fatty acids (SCFAs) production by high performance liquid chromatography. TLC profile evinced the ability of these two strains to utilize low molecular weight galactooligosaccharides (GOS) and fructooligosaccharides (FOS), as only the upper low molecular weight fractions were disappeared from cell-free-supernatants (CFS). Enhanced β-galactosidase activity correlated with galactose accumulation in residual CFS of GOS displayed GOS utilization ability. Both the strains exhibited AMA against *E. coli* and *Staph. aureus* and high SCFAs production in the presence of prebiotic, suggesting their synbiotic potential. Thus, *W. parameseteroides* strains FX5 and FX9 exhibit potential probiotic properties with prebiotic utilization and can be taken forward to evaluate synergistic synbiotic potential in detail.

Keywords *Weissella parameseteroides* · Probiotics · Prebiotics · Antimicrobial activity · Short-chain fatty acids

Introduction

Lactic acid bacteria (LAB) are predominantly present in various fruits, vegetables, and fermented foods, and in addition, they are present as normal flora of gastrointestinal tract (GIT) and vagina of humans and animals [1]. LAB produce lactic acid as a major end product of carbohydrate metabolism along with other end products such as acetic acid and CO_2 during heterolactic fermentation. Many LAB are characterized as potential probiotics, which are living microorganisms that when administered in adequate amounts confer health benefits to the hosts [2]. They may play a crucial role in modulating the physiological functions of the gut by improving digestion and by inhibiting the growth of pathogens, thereby preventing gastrointestinal infections [1, 3]. Strains of lactobacilli such as *Lactobacillus plantarum*, *Lact. fermentum*, and *Lact. rhamnosus* [4, 5] and certain other LAB such as

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Streptococcus thermophilus, *Lactococcus lactis*, *Weissella paramesenteroides*, and *W. cibaria* are known examples of LAB exhibiting potential health benefits [1, 6].

Prebiotics are non-digestible carbohydrates (NDCs) that selectively stimulate the growth and/or activity of host microorganisms in the colon and confer a beneficial effect on host [7]. NDCs are resistant to hydrolysis by salivary and intestinal digestive enzymes but are sensitive to hydrolysis by enzymes of colon bacteria. Most NDCs contain 3 to 10 sugar moieties, although the degree of polymerization (DP) could go up to 60 for some NDCs, like chicory inulin or down to 2 for some NDCs like lactulose [8]. Short-chain fatty acids (SCFAs) are one of the antimicrobial metabolites produced by NDC utilizing probiotic bacteria that act against the pathogenic microbes [9]. Galactooligosaccharides (GOS), fructooligosaccharides (FOS), and xylooligosaccharides (XOS) are well-known prebiotics [10].

A synbiotic is defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and/or activity of beneficial microorganisms in the gut [11]. Recently, it has gained considerable attention owing to the ability to enhance the probiotic bacteria in the GIT by selectively stimulating the growth and/or by activating the metabolism of indigenous gut microflora and thereby conferring health benefits [11].

Weissella species have been isolated from various fruits such as watermelon and other citrus fruits such as grapes and tomatoes exhibiting good acidification potential, antimicrobial activity (AMA), and exopolysaccharide production [12]. However, there is scarce information available on probiotic characterization of *Weissella* species and their ability to utilize various prebiotics. Thus, it is noteworthy to explore *Weissella* species for their potential application as probiotics and to investigate their prebiotic utilization ability.

The present study aimed to evaluate fruit origin *W. paramesenteroides* strains for their functional and safety aspects, which are necessary for their application as probiotic strains. Further the selected potential strains were evaluated for their prebiotic utilization ability and SCFAs production profile.

Materials and Methods

Chemicals and Media

De Man Rogosa Sharpe (MRS) broth, FOS (DP – between 2 and 60, with an average DP > 10), nutrient broth (NB), brain heart infusion (BHI), *o*-nitrophenyl-*β*-D galactopyranoside (ONPG), and 0.22-μm cellulose nitrate membrane filter were purchased from Himedia, Mumbai, India. Sodium taurocholate (ST) was purchased from Loba Chemie, Mumbai, India. XOS was provided by Sweet Town Biotech, Taiwan, and Vivinal GOS (DP – 2–6) was a kind gift from FrieslandCampina, Amersfoort, the Netherlands. Mucin type III from porcine stomach was purchased from Sigma-Aldrich, St. Louis, USA. About 96-well microtiter plates (MTP) were purchased from Tarson, Kolkata, India. All chemicals were of analytical grade. MRS broth with 2% glucose without additional components was used as normal MRS broth in all tests if otherwise stated. MRS basal broth (MRS-BB) contained all the components of MRS medium except beef extract and glucose from normal MRS medium.

Isolation, Screening, and Molecular Identification of *Weissella*

Various fresh fruits (Sapota, Cherry, Banana, Orange, and Plum) collected from local market were thoroughly washed with sterile distilled water to remove surface impurities and were smashed in sterile condition. One gram of smashed fruit samples were added into MRS-BB supplemented with 0.5% ST and 0.5% filter-sterilized GOS or FOS or XOS as sole carbon source and incubated at 37 °C for 48 h. Appropriate dilutions from the enriched samples prepared in phosphate buffer saline (PBS: 0.1 M, pH 7.0) were plated on MRS agar and further incubated at 37 °C for 48 h. Various spindle-shaped colonies were inoculated in MRS broth and incubated at 37 °C for 48 h. These isolates were further screened for gram reaction and catalase test.

Screening of 12 isolates was performed based on the ability to grow in the presence of low pH, ST, NaCl and phenol, and AMA against enteropathogens. To evaluate growth of 12 LAB isolates in stress conditions, cells (OD_{600nm} = 1.0) were inoculated in 2 ml normal MRS broth, MRS broth with either ST (0.5, 1.0%) or NaCl (2.0, 4.0%) or adjusted pH (3.0, 2.5) or phenol (0.2, 0.4%). All the tubes were incubated at 37 °C for 24 h, and the growth was determined by measuring the OD_{600nm} using single beam UV-visible spectrophotometer (EI, Hyderabad, India). Further AMA against four pathogens, namely, *Escherichia coli* MTCC1697, *Salmonella typhi* MTCC98, *Staphylococcus aureus* MTCC1144, and *Shigella sp*. (clinical strain), was evaluated by spot inoculation test according to Pithva et al. [13].

For molecular identification, genomic DNA of five selected isolates was extracted using bacterial DNA isolation kit as per the manufacturer's instructions (Zymo Research, CA, USA). The quality of isolated DNA was checked on 0.8% agarose gel. 16S rRNA gene amplification product was generated from these DNA samples using universal primers UNI 8F (5 -AGAGTTTGATCCTGGCTGAG-3) and UNI 1492R (5 -GTTACCTTGTTACGACTT-3) (Eurofins, Bengaluru, India). Conditions used for the amplification were 98 °C for 2 min, 98 °C for 20 s, 56 °C for 30 s, 72 °C for 80 s, and 72 °C for 5 min (Veriti Thermal Cycler, Applied Biosystems, Waltham, USA) [14]. The PCR products obtained were

checked for the quality on 1% agarose gel and were sent for sequencing, and the sequences obtained were analyzed for homology on National Center for Biotechnology Information (NCBI) database for species-level identification. Nucleotide sequences obtained were submitted to the GenBank with the following accession numbers: FX1-MN252462, FX2- MN252463, FX5- MN252464, FX9-MN252465, and FX12- MN252466.

Preparation of Cell Suspension of LAB

The pure isolates of selected LAB from glycerol stock were inoculated into MRS broth and were subcultured three times in the same medium before being used further in the experiments. For the preparation of cell suspension, 24-h grown cultures were harvested by centrifugation (5000 × *g*, 15 min), washed twice, and resuspended in PBS. Each time OD_{600nm} of cultures was adjusted to 1.0 or 0.5 according to the experimental requirements.

Functional Characterization

Viability in Low pH and ST

To evaluate viability in low pH and ST, *Weissella* strains (OD_{600nm} = 1.0) were inoculated in MRS broth adjusted to pH 3.0 or 2.5 with 1 N HCl and MRS broth supplemented with 0.5 or 1.0% ST and were incubated at 37 °C for 1 and 3 h, respectively. About 100 μl of culture broth was harvested from these tubes and serially diluted up to 10^{-7} dilutions; last three dilutions were plated on MRS agar and incubated at 37 °C for 48 h to determine the viable cell counts, expressed as log CFU/ml [15]. The viability of cells determined in normal MRS broth after 1 and 3 h served as positive controls.

Salt Aggregation Test (SAT) and Autoaggregation

The cell surface hydrophobicity (CSH) of *Weissella* strains was determined by SAT [16]. Briefly, a 10-μl aliquot of a washed cell suspension prepared in PBS was mixed on a glass slide with 10 μl of ammonium sulfate (pH 6.8) of various molarities (0.02, 0.2, 0.8, 1.6, 3.2, and 4.0 M). The molarity at which the cells caused aggregation was recorded as a positive result. Strains with SAT values of 0.02 M and 4.0 M were termed as autoaggregating (AA) and non-AA, respectively. Further, the autoaggregation ability of *Weissella* strains was also determined as described by Campana et al. [17]. Briefly, *Weissella* strains (OD_{600nm} = 1.0) were added in 2-ml phosphate buffer (PB) (0.07 M, pH 7.0) and incubated at 37 °C. An aliquot of 0.1 ml sample from upper surface was withdrawn at time 0 and 4 h and was mixed with 0.9 ml PB to measure OD_{600nm}. The autoaggregation (%) was calculated as $[(OD_0 - OD_t)/OD_0] \times 100$, where OD_0 represents OD_{600nm} at 0 h and OD_t represents the OD_{600nm} of upper cell suspension after 4 h.

Biofilm Formation

To study bile-induced biofilm formation, *Weissella* strains were inoculated (OD_{600nm} = 1.0) in each well of 96-well MTP filled with either 200 μl MRS broth or MRS broth with 0.5 or 1.0% ST and incubated at 37 °C for 72 h. Further biofilm formation assay was performed according to Ambalam et al. [18]. Briefly, media was decanted from the MTP after 72 h, and wells were washed thrice with sterile distilled water. Surface-adhered bacterial cells were stained with 0.1% crystal violet (CV) prepared in isopropanol-methanol-PBS (1:1:18 *v*/v) for 30 min. Plates were again washed thrice with sterile distilled water and air dried, and the bound CV from the adhered cells was extracted with 200 μl of dimethyl sulfoxide, and OD_{570nm} of each well was measured using MTP reader (Thermofisher, Waltham, USA). The amount of surface-bound CV (μg/well) was determined using a standard curve of CV.

In vitro Adhesion to Mucin

Adhesion assay was performed in 96-well polystyrene MTP using mucin as matrix according to Valeriano et al. with slight modifications [19]. The wells were coated with 300 μl of mucin (0.5 mg/ml) dissolved in Dulbecco's PBS (pH 7.0), placed overnight at 4 °C, and were washed thrice with PBS (0.07 M, pH 7.0). To study mucin adherence, washed bacterial cell suspension (OD_{600nm} = 1) was added to mucin-coated wells and incubated at 37 °C for 90 min. After incubation, wells were again washed five times with PBS to remove unbound bacteria. About 300 μl of 0.05% (*v*/v) triton X-100 prepared in PBS was then added to detach bacterial cells adhered to mucin. The viable cell count was performed after plating appropriate dilutions on MRS agar, expressed as log CFU/ml, and was compared with log CFU/ml before adhesion to calculate the relative adhesion as described below.

$$\%\text{Relative adhesion} = \frac{\log \text{CFU/ml after adhesion}}{\log \text{CFU/ml before adhesion}} \times 100$$

Safety Aspects

Weissella strains were studied for the standard food microbial safety aspects. For antibiotic susceptibility test, a 100-μl cell suspension was plated with molten MRS agar (1%), and octadiscs impregnated with specific antibiotics (Himedia, Mumbai, India) were positioned on the MRS agar plates and incubated at 37 °C for 48 h. Zone of inhibition (millimeters) was recorded for each strain. Hemolytic activity was checked

by streaking cultures of *Weissella* strains on MRS agar plates containing 5% human blood. DNase activity was tested by HCI-DNA precipitation method [20]. Briefly, *Weissella* strains were streaked on DNase agar pates (15 g l^{-1} tryptone, 5 g l^{-1} soya peptone, 2 g l^{-1} DNA, 5 g l^{-1} NaCl, 20 g l^{-1} agar powder) and were incubated at 37 °C for 48 h. The clear zone around the colony after flooding the DNase agar plates with 1.0 N HCI was considered as positive for DNase activity. Tyrosine decarboxylase activity was tested by streaking cultures of *Weissella* strains on MRS agar plates containing 0.1% tyrosine. The clear zone around the colony was considered as positive result [13]. *Bacillus megaterium* (clinical strain), *Staph. aureus* MTTC1144, and *Enterococcus faecalis* (clinical strain) served as positive controls for hemolytic activity, DNase activity, and tyrosine decarboxylase activity, respectively.

Prebiotic Utilization Studies

Prebiotic Utilization and Prebiotic Score (PS)

Weissella strains were evaluated for prebiotic utilization and PS as described earlier by Kondepudi et al. [15]. Briefly, *Weissella* strains (OD_{600nm} = 0.5) were inoculated in 5-ml MRS-BB supplemented with 1% prebiotics (GOS or FOS or XOS) and incubated at 37 °C for 72 h. MRS-BB with 1% glucose was used as positive control. Prebiotic utilization was determined by measuring growth of cells (OD_{600nm}) and pH of cell-free supernatants (CFSs) at an interval of 24, 48, and 72 h.

A PS is the highest growth achieved by a strain in the presence of MRS-BB supplemented with prebiotics relative to their growth in the presence of MRS-BB supplemented with glucose that was considered as 100% [15]. The PS was determined using the formula: PS = (A/B) × 100%, where A and B are the mean OD_{600nm} values of a strain grown in the presence of each of the prebiotics (GOS, FOS, or XOS) or glucose, respectively, after 48 h of growth.

β-Galactosidase Activity

β-galactosidase activity of *Weissella* strains was determined using the chromogenic substance ONPG by MTP assay. Cells of *Weissella* strains (OD_{600nm} = 1.0) were inoculated in MRS-BB supplemented with either 1% GOS or 1% glucose and incubated at 37 °C for 24 h. Cells were harvested from the broth and washed twice with PBS (70 mM, pH 7.0). The reaction mixture for β-galactosidase assay contained a total of 200-μl system with 50 μl (OD_{600nm} = 0.5) of bacterial cell suspension prepared in Z-buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCL, 1 mM $MgSO_4{\cdot}7H_2O$, and 50-mM β-mercaptoethanol) and 5 μl of 0.1% sodium dodecyl sulfate for the cell lysis; system was mixed well and incubated at 37 °C for 30 min. Later, 55 μl 10 mM ONPG (prepared in 70 mM PBS, pH 7.0) and 90 μl same PBS were added. The reaction mixture was then incubated for 15 min and stopped by adding 200 μl of 1 M Na_2CO_3. The absorbance was measured at OD_{420nm} and OD_{520nm} using MTP reader (Thermofisher, Waltham, USA). β-galactosidase activity was expressed in Miller Units/ml as described earlier [21].

AMA of CFSs and Extracellular Protein Concentrate (EPC) by MTP Assay

Preparation of CFSs and EPC

To prepare CFSs, *Weissella* strains (OD_{600nm} = 1) were inoculated in MRS-BB supplemented with either 1% glucose or GOS or FOS and were incubated at 37 °C for 24 h. Further, CFSs were obtained by centrifugation (5000 × *g*, 15 min, 4 °C), followed by filtration through 0.22 μm filter. EPC was prepared from CFS filtrate obtained from 24-h old culture of *W. paramesenteroides* FX5 grown in MRS broth at 37 °C. CFS obtained after centrifugation (10,000×*g*, 15 min, 4 °C) was passed through a 0.22-μm membrane filter, and protein present in the CFS filtrates was precipitated by ammonium sulfate to 80% saturation; afterwards the protein precipitates were collected by centrifugation (10,000 × *g*, 15 min, 4 °C) and dissolved in a minimum amount of acetate buffer (10 mM, pH 5.5) and was labeled as EPC. Clear EPC obtained upon centrifugation was further desalted by dialyzing it against acetate buffer (10 mM, pH 5.5) using 1-kDa membrane bag (Spectrum, New Brunswick, NJ, USA).

AMA of CFSs and EPC Against *E. coli* and *Staph. aureus*

AMA of CFSs and EPC was determined against *E. coli* and *Staph. aureus* using NB and BHI, respectively. CFSs and EPC were diluted to 1:5 using growth media, and 300 μl of each dilution was added in the well of sterile MTP. *E. coli* and *Staph. aureus* were inoculated with an initial OD_{600nm} equivalent to 0.4 and incubated at 37 °C for 24 h. Growth was measured after 24 h at OD_{620nm} using a MTP reader (Thermofisher, Waltham, USA). Percent inhibition of test pathogens was determined as described earlier by Ambalam et al. [22].

Analysis of Prebiotic Utilization by Thin Layer Chromatography (TLC)

TLC was used to determine residual GOS or FOS present in the CFSs of two selected strains FX5 and FX9 after 24, 48, and 72 h growth. MRS-BB supplemented with either 1% FOS or GOS was used as control, for the detection of residual FOS

and GOS, respectively. About 1.5 μl CFSs and control samples were applied to a pre-coated silica gel 60 (F254, 0.25 mm) plate (Merck, Darmstadt, Germany). Butanol, ethanol, acetic acid, and distilled water (1.0:0.3:0.3:0.15) and butanol, acetic acid, and distilled water (2.0:1.0:1.0) were used as solvent systems for analyzing residual FOS and GOS, respectively. For detection, plates were sprayed with 20% sulfuric acid consisting of 0.5% 1-naphthol prepared in ethanol, and TLC plates were placed at 110 °C for 15 min in hot air oven.

Lactic Acid and SCFAs Production Profile

CFSs from two selected strains FX5 and FX9 grown in MRS-BB supplemented with 1% FOS or GOS or glucose for 48 h were analyzed for the production of lactate and SCFAs by HPLC (Waters 600, Waters, Milford, USA) using Agilent Hi-Plex H (7.7 × 300 mm, 8 μm) (Agilent, Santa Clara, CA, USA) column and 1 mM H_2SO_4 as a mobile phase (0.6 ml/min). MRS broth without any carbon source served as negative control. Lactic acid and SCFAs, namely, acetic acid, propionic acid, butyric acid, and formic acid, were quantitatively determined from their standard curves.

Statistical Analysis

All test values are means of ± standard deviation (SD) performed in triplicate ($n = 3$). Statistical differences among the results were analyzed by one-way analysis of variance (ANOVA) using a Minitab software (version 14.0, Minitab Inc., USA). P values < 0.05 were considered as significant. The comparison made for the statistical analyses is indicated in the legends of figures.

Results

Isolation, Screening, and Molecular Identification of *Weissella*

Twelve out of 51 isolates showed typical characteristics of LAB (catalase negative, gram positive, irregular short rods) and were screened for their growth in the presence of low pH (pH 3.0, 2.5), ST (0.5, 1.0%), NaCl (2.0, 4.0%), and phenol (0.2, 0.4%) along with AMA against enteropathogens, namely, *E. coli*, *Shigella sp*., *Salm. typhi*, and food-spoilage organism *Staph. aureus*. Isolates showed growth in MRS broth with ST (0.5 or 1.0%), NaCl (2.0 or 4.0%), and 0.2% phenol, at par with the growth in normal MRS broth. Growth of isolates in MRS broth with pH (3.0 or 2.5) and 0.4% phenol was reduced to 50% compared to normal MRS broth (Table S1). However, upon reinoculation to a fresh normal MRS broth, the cells could restore the normal growth, which indicates that these cells have certain type of regulatory mechanism to evade the stress conditions of low pH and phenol. Additionally, the 50% reduction in growth might be due to the slower growth rate of these cells in the presence of low pH and phenol than to the detrimental effect of these compounds on these cells. All these 12 LAB isolates exhibited AMA against *E. coli*, *Shigella sp*., *Salm. typhi*, and *Staph. aureus* (Table S2). Among these 12 isolates, only 4 isolates, i.e., FX1, FX2, FX9, and FX10, exhibited higher AMA against *Staph. aureus*, whereas FX10, FX11, and FX12 showed higher AMA against *Salm. typhi*, and FX1, FX2, FX5, and FX9 showed high AMA against *E. coli* and *Shigella sp*. Isolates FX2, FX5, FX9, and FX12 exhibited prominent AMA against all the four pathogens. On the basis of these two preliminary screening tests, five LAB isolates, namely, FX1, FX2, FX5, FX9, and FX12, were selected for further evaluation of probiotic characteristics, safety aspects, and prebiotic utilization ability. 16S rDNA sequencing of these five isolates revealed that the FX1 exhibited 97% similarity, while FX2, FX5, FX9, and FX12 exhibited 99% similarity with the 16S rDNA sequence of *Weissella paramesenteroides*.

Functional Characterization

Viability in Low pH and ST

The viability of five *W. paramesenteroides* strains was studied after exposure to pH (pH 3.0, 2.5) and ST (0.5, 1.0%) (Table 1). Two strains, FX2 and FX12, showed nonsignificant log CFU reduction in the presence of 0.5% ST ($P < 0.05$), while strains FX1, FX5, and FX9 showed marginal reduction of ca. < 0.3 log CFU. Increase in ST concentration from 0.5 to 1.0% did not significantly affect the viability of strains, while reduction of pH from 3.0 to 2.5 showed the significant reduction in viability of the strains. At pH 3.0, strains showed reduction of ca. < 0.22 log CFU, and at pH 2.5, strains showed reduction of ca. < 1 log CFU.

Salt Aggregation Test (SAT) and Autoaggregation

Cell surface hydrophobicity (CSH) of five *W. paramesenteroides* strains was evaluated by SAT and autoaggregation assay (Table 2). All five *W. paramesenteroides* strains exhibited low SAT values (≥ 0.02 M) implicating high CSH. Autoaggregation ability of five *W. paramesenteroides* isolates, measured after 4 h, was within the range of 22–36%. FX1, FX2, and FX5 showed up

Table 1 Viability (log CFU/ml)[a] of *Weissella* strains in the presence of normal MRS broth, MRS broth with ST (0.5, 1%) after 3 h, and MRS broth with low pH (3.0, 2.5) after 1 h of incubation at 37 °C

Weissella strains	MRS + ST			MRS with low pH		
	0% ST	0.5% ST	1% ST	pH 7.0	pH 3.0	pH 2.5
FX1	8.62 ± 0.01	8.51 ± 0.06*	8.47 ± 0.06*	8.58 ± 0.03	8.41 ± 0.08*	7.07 ± 0.17*
FX2	8.81 ± 0.09	8.75 ± 0.16	8.73 ± 0.36	8.23 ± 0.6	8.12 ± 0.6	6.59 ± 0.5*
FX5	8.74 ± 0.05	8.62 ± 0.07*	8.60 ± 0.05*	8.63 ± 0.04	8.46 ± 0.05*	7.15 ± 0.13*
FX9	8.52 ± 0.01	8.12 ± 0.07*	8.11 ± 0.04*	8.47 ± 0.05	8.32 ± 0.08*	7.58 ± 0.07*
FX12	9.73 ± 0.37	9.40 ± 0.31	9.31 ± 0.45	8.56 ± 0.03	8.46 ± 0.04*	7.51 ± 0.16*

[a] Values are mean ± SD of three replicates. * Significantly differs relative to normal MRS broth ($P < 0.05$)

to 26% AA, and FX9 and FX12 showed 27 and 36% AA, respectively.

Biofilm Formation

Bile-induced biofilm formation of five *W. paramesenteroides* strains was evaluated by MTP assay (Fig. 1). All the strains except FX12 exhibited significantly ($P \leq 0.05$) enhanced biofilm formation in the presence of 0.5 and 1.0% ST compared to normal MRS broth, while FX12 showed decreased biofilm formation in the presence of 1% ST compared to normal MRS broth. FX1 and FX9 showed higher biofilm formation, followed by FX5 and FX2.

In Vitro Adhesion to Mucin

In vitro adhesion ability of five *W. paramesenteroides* strains to mucin was evaluated using porcine stomach mucin (Table 2). Three strains FX1, FX2, and FX5 showed up to 75% relative adhesion to mucin, while FX9 and FX12 exhibited 56% relative adhesion to mucin (Table 2).

Safety Aspects

Safety aspects of five *W. paramesenteroides* strains were evaluated by determining the antibiotic susceptibility profile, hemolytic activity, DNase activity, and tyrosine decarboxylase activity. All the five strains exhibited similar antibiotic susceptibility profile (Table S3). None of the strains showed resistance to the studied antibiotics except vancomycin, which is a common characteristic of *Lactobacillaceae* members encoded by the plasmids. Strains were susceptible to antibiotics having different mode of action like inhibitors of cell wall synthesis (cefotaxime, amoxicillin, and ampicillin), inhibitors of protein synthesis (amikacin, gentamicin, and erythromycin), and nucleic acid synthesis (ciprofloxacin and levofloxacin). Strains were also susceptible to cephalexin (first-generation antibiotics), cefuroxime (second-generation antibiotics), cefotaxime (third-generation), and cefepime (fourth-generation). None of the strains showed hemolytic reaction on blood agar. Strains were neither DNase positive nor tyramine producing, as there was no zone of clearance surrounding the colony on DNase plate or tyrosine decarboxylase medium, suggesting the absence of DNase or tyramine production, respectively.

Table 2 In vitro adhesion properties (measured in terms of SAT, %autoaggregation after 4 h, and %relative adhesion to mucin), prebiotic scores, and β-galactosidase activity of *Weissella* strains

Weissella strains	SAT values[b] (M)	% Autoaggregation[a]	%Relative adhesion to mucin[a]	Prebiotics score[ac] (%)			β-galactosidase activity[d] (Miller Units/ml)	
				GOS	FOS	XOS	GOS	Glucose
FX1	≥0.02	26 ± 1.2	71 ± 3	64 ± 0.2	80 ± 0.7	50 ± 1.1	5194 ± 32*	178 ± 16
FX2	≥0.02	24 ± 0.4	73 ± 4	52 ± 3.3	70 ± 0.8	48 ± 0.6	1794 ± 44*	44 ± 16
FX5	≥0.02	22 ± 0.5	75 ± 2	70 ± 1.5	75 ± 0.4	28 ± 0.5	4253 ± 92*	262 ± 21
FX9	≥0.02	27 ± 0.3	56 ± 1	71 ± 2.8	73 ± 2.3	51 ± 3.9	1892 ± 22*	38 ± 13
FX12	≥0.02	36 ± 0.8	52 ± 1	57 ± 2.6	56 ± 3.5	50 ± 3.1	1738 ± 17*	25 ± 5

[a] Values are mean ± SD of three replicates

[b] SAT values were scored as lowest molar concentration of ammonium sulfate at which cells tend to form visible aggregates

[c] Prebiotic scores of *Weissella* strains grown in presence of 1% prebiotics after 48 h of incubation at 37 °C

[d] β-galactosidase activity of *Weissella* strains grown in MRS-BB supplemented with either 1% GOS or 1% glucose expressed in Miller Units/ml after 48 h of incubation at 37 °C. * β-galactosidase activity increased significantly ($P < 0.05$) in the presence of GOS compared to glucose

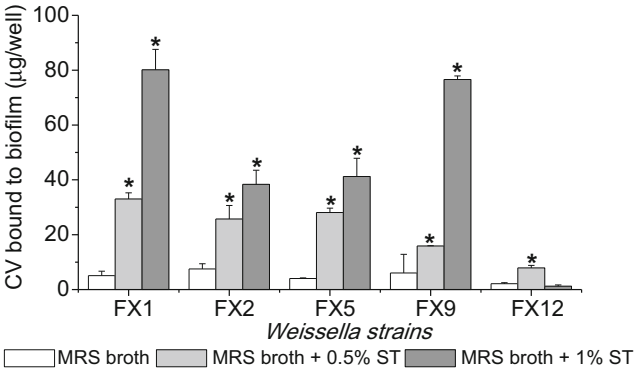

Fig. 1 Biofilm formation by *Weissella* strains grown in normal MRS broth and MRS broth supplemented with either 0.5 or 1.0% ST after 72 h of incubation at 37 °C. Error bars indicate SD of three replicates. *Significance ($P < 0.05$) was expressed in comparison with the normal MRS broth

Prebiotic Utilization Studies

Prebiotic Utilization and PS

All the strains showed varying degree of growth, pH drop, and PS when grown in the presence of different prebiotics, viz., FOS or GOS or XOS measured after 24, 48, and 72 h of growth. Strains showed maximum PS and pH drop at 48 h of growth. Strains FX1, FX5, and FX9 showed high PS (Table 2), with pH drop up to pH 4.9 of the medium in the presence of FOS or GOS (Fig. 2). FX1 showed highest PS in the presence of FOS (up to 80%), followed by GOS (up to 64%). FX5 and FX9 showed up to 75% PS in presence of either GOS or FOS. The PS of the five strains was less than 50% in the presence of XOS with the pH drop of CFS up to pH 6.0.

β-Galactosidase Activity

β-galactosidase activity of five *W. paramesenteroides* strains was evaluated after the growth in the presence of glucose or GOS (Table 2). Compared to the other isolates, two strains FX5 and FX1 exhibited higher β-galactosidase activity with either glucose or GOS. All the strains exhibited enhanced β-galactosidase activity in the presence of GOS compared to glucose ($P < 0.05$). With glucose, FX5 and FX1 exhibited 262 and 178 Miller Units/ml β-galactosidase activity, respectively, while with GOS, these strains exhibited enhanced β-galactosidase activity up to 4253 and 5192 Miller Units/ml, respectively.

AMA of CFSs and EPC by MTP Assay

CFSs of five strains obtained by growing in the presence of either 1% glucose or FOS or GOS were evaluated for AMA against *E. coli* and *Staph. aureus* at 1:5 dilution (Fig. 3). Against *E. coli* AMA was higher in the presence of FOS, followed by glucose and GOS. With FOS AMA was significantly higher ($P < 0.05$) than the rest of the carbon source. On the contrary, against *Staph. aureus*, AMA was higher in the presence of glucose, followed by FOS and GOS. Further, AMA of dialyzed EPC (protein concentration- 221 µg/ml) from selected strain FX5 with 1:5 dilution of EPC was 20 ± 1% and 37 ± 2% against *E. coli* and *Staph. aureus*, respectively. Acidic

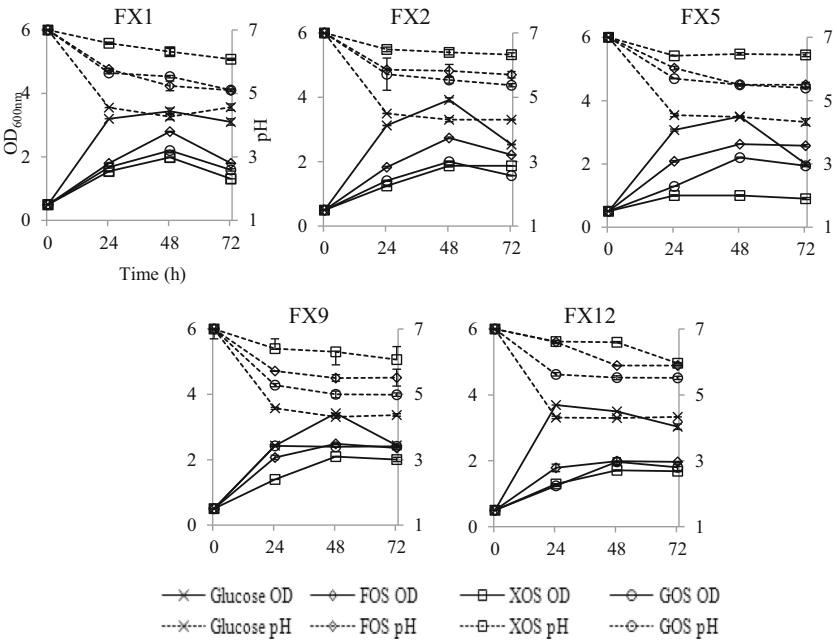

Fig. 2 Growth (OD_{600nm}) and pH profile of *Weissella* strains after 24, 48, and 72 h of incubation at 37 °C in MRS-BB supplemented with either 1% glucose or 1% prebiotics. Error bars indicate SD of three replicates

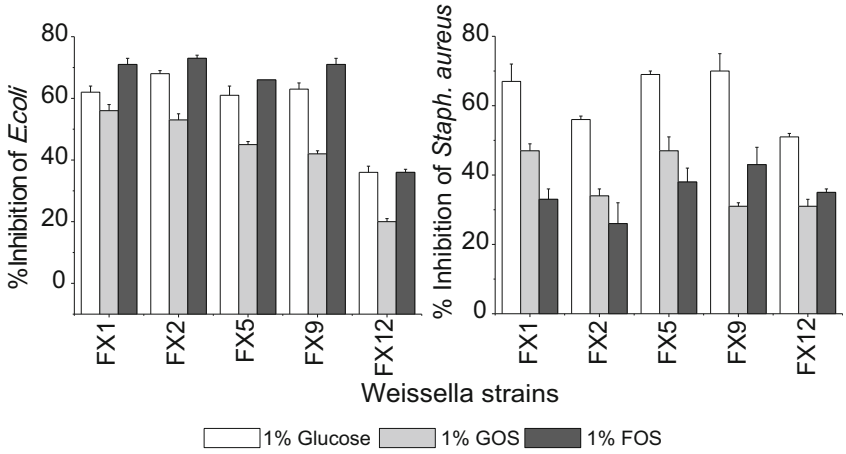

Fig. 3 AMA of CFSs obtained by growing *Weissella* strains at 37 °C for 24 h in MRS-BB supplemented with either 1% glucose or 1% GOS or 1% FOS. CFSs were diluted to 1:5 in sterile NB and BHI for *E. coli* and *Staph. aureus*, respectively. Error bars indicate SD of three replicates

CFSs exhibited higher AMA compared to dialyzed EPC ($P < 0.05$).

Analysis of Prebiotic Utilization with TLC

TLC profile of residual GOS or FOS present in the CFS of two selected strains FX5 and FX9 revealed ability of both the strains to utilize low molecular weight GOS or FOS, as the intensity of upper portion of TLC was reduced prominently, while the high molecular weight oligosaccharides were remained unutilized till the end of 72 h. FX9 showed more utilization of FOS compared to FX5 (Fig. 4B), while FX5 showed more utilization of GOS compared to FX9 (Fig. 4A). Moreover, TLC profile of GOS of both the strains showed the accumulation of galactose in the CFSs during the growth phase of 24, 48, and 72 h. Furthermore, the intensity of GOS or FOS spot was decreased with increased growth phase.

Lactic Acid and SCFAs Production Profile

FX5 and FX9 produced varying amounts of lactic acid and SCFAs when grown in the presence of either glucose or GOS or FOS as a sole carbon source (Table 3). Lactic acid production was predominant when strains were grown in in the presence of glucose, while SCFAs production was increased when strains were grown in in the presence of prebiotics (GOS or FOS). FX5 showed higher SCFAs production in the presence of FOS or GOS than glucose, whereas FX9 showed more SCFAs production in the presence of FOS compared to glucose and GOS. In the presence of FOS, both the strains showed higher production of various organic acids in the decreasing order of acetic acid > lactic acid > propionic acid > formic acid > butyric acid; on the contrary in presence of GOS, only FX5 showed higher production of various organic acids in the decreasing order of lactic acid > acetic acid > formic acid > propionic acid > butyric acid.

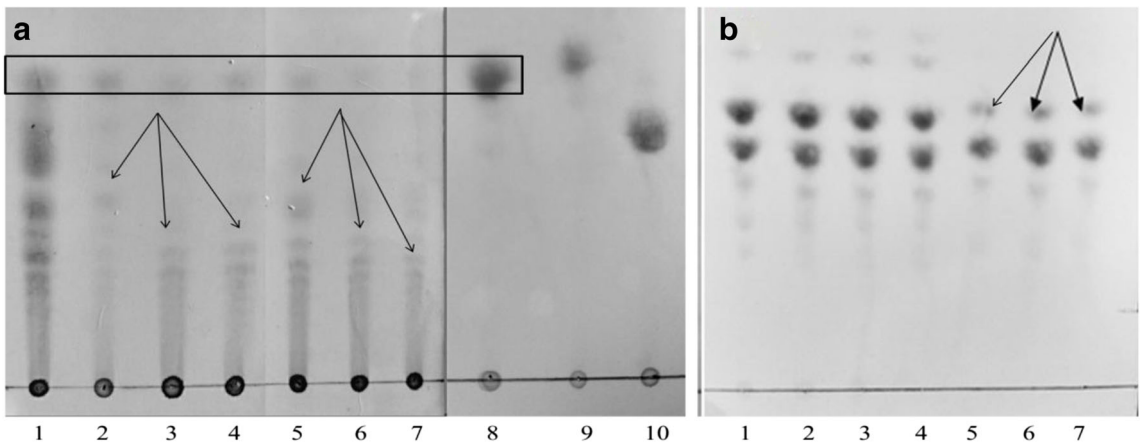

Fig. 4 TLC analysis of 24, 48, and 72 h CFSs obtained after growth on (4A) 1% GOS and (4B) 1% FOS. (4A) lane 1: MRS-BB with 1% GOS; lane 2: 24 h CFS of FX5; lane 3: 48 h CFS of FX5; lane 4: 72 h CFS of FX5; lane 5: 24 h CFS of FX9; lane 6: 48 h CFS of FX9; lane 7: 72 h CFS of FX9; lane 8: 0.1% galactose; lane 9: 0.1% glucose; lane 10: 0.1% lactose. (4B) lane 1: MRS-BB with 1% FOS; lane 2: 24 h CFS of FX5; lane 3: 48 h CFS of FX5; lane 4: 72 h CFS of FX5; lane 5: 24 h CFS of FX9; lane 6: 48 h CFS of FX9; lane 7: 72 h CFS of FX9. Arrows indicate disappearance of old spot from CFS samples compared to control. Spots indicated by square box show accumulation of galactose in CFSs after GOS utilization

Table 3 Lactic acid and SCFAs production (ppm) of two selected *Weissella* strains on MRS-BB supplemented with either 1% glucose or 1% GOS or 1% FOS after 48 h of incubation at 37 °C

Lactic acid and SCFAs concentration (ppm)	FX5			FX9		
	Glucose	GOS	FOS	Glucose	GOS	FOS
Lactic acid	3432	5272	3610	6421	2123	4491
Formic acid	317	1047	1800	412	337	345
Acetic acid	863	6107	5370	2196	2635	5361
Propionic acid	863	966	2030	3176	1106	2696
Butyric acid	158	16	202	190	113	186
Total acid and SCFAs	5633	13,408	13,012	12,395	6314	13,079

Discussion

Fruits and vegetables are good sources of non-digestible dietary prebiotic fibers and probiotics. Moreover, incorporation of fruits and vegetables in human diet would help to meet the daily requirement of dietary prebiotic fibers which are needed for a healthy gut [23]. Since they are rich in prebiotic components and they are potential vectors of probiotics, in the present study, fruits were selected as source for the isolation of new potential probiotic strains that can also utilize prebiotics. A total of 12 LAB were isolated from different fruits using MRS-BB supplemented with different prebiotics (GOS or FOS or XOS). Out of these 12 LAB isolates, 5 robust strains, namely, FX1, FX2, FX5, FX9, and FX12, were selected based on qualification of the first line of criterion to be considered as probiotics, i.e., growth and survival in (i) low pH present in gastric tract; (ii) the presence of bile (ST) in small intestine; (iii) presence of phenol, i.e., produced during putrefaction of aromatic amino acids by intestinal bacteria; (iv) presence of high salt concentration, i.e., vital for food preservation during fermentation process; and additionally these isolates were studied for the (v) AMA against enteropathogens and food poisoning microorganism.

Molecular identification revealed the sequence homology of these five isolates with *W. paramesenteroides*, earlier known as *Leuconostoc paramesenteroides* and later reclassified in the new genus *Weissella* [24]. Since the majority of the reports on *W. paramesenteroides* have been focused on novel bacteriocin production [6, 25], the present study is slightly away from bacteriocin production and focused towards the characterization of putative probiotic properties of *W. paramesenteroides* and prebiotic utilization ability of *W. paramesenteroides*.

Resistance to low pH and bile salts are important for the survival and colonization of LAB in the GIT. All the five strains of *W. paramesenteroides* retained viability in the presence of 1.0% ST for 3 h and at pH 2.5 for 1 h, representing that they might harbor acid tolerance-associated genes encoding F1F0 ATP synthase as reported earlier for *W. jogaejeotgali* strain FOL0[1] [26] and *W. cibaria* strains [27]. Furthermore, high percent viability (up to 85%) of strains in low pH might be due to their adaptive mechanism that they might have followed for their survival, which could be ascribed to their isolation source, as fruits belonging to citrus family naturally possess low pH due to their acid content. On the contrary, *W. cibaria* strains have been reported earlier for comparatively low survival, i.e., up to 68% viability at low pH [28]. Some *Lactobacillus* strains, such as *Lact. plantarum* WCFS1 and *Lact. reuteri*, showed tolerance to bile due to active bile salts efflux mechanism or changes in the cell membrane and cell wall composition [29, 30]. In the present study, strains retained up to 90% viability in the presence of 1.0% ST for 3 h. However, mechanism involved in bile tolerance ability of present studied *W. paramesenteroides* is remained unexplored.

Probiotic bacteria should also be able to adhere to the mucosal surfaces for their successful colonization and longer persistent in the GIT [31, 32]. *Weissella* strains showed lower SAT values (0.02 M) and autoaggregation up to 36% after 4 h, implicating the hydrophobic nature of their cell surface, which may facilitate the colonization of these strains in the gut. However, detailed studies are needed to be undertaken to characterize cell surface proteins in the *W. paramesenteroides* as it is investigated in-detail in *Lactobacillus* strains such as *Lact. acidophilus* [33].

Biofilm formation by LAB may promote adherence and thus their colonization and longer persistence in the mucosa of the host intestine, which creates a competition for nutrition and lodging of enteropathogens on the host mucosal surfaces. Additionally, the biofilms formed around these bacteria increase the survival of these colonized bacteria in adverse conditions. The formation and development of a biofilm are affected by multiple factors, including the genetics of the bacterial strain, cell surface properties, and environmental parameters such as pH and nutrient concentration [34]. The present study reports bile-induced biofilm formation by *W. paramesenteroides* strains. Such bile-induced biofilm formation is reported earlier in lactobacilli and bifidobacteria [18, 35], and to the best of our knowledge, it is the first report on bile-induced biofilm formation by *W. paramesenteroides*. However, the exact molecular mechanism involved in

the stress-induced biofilm formation by *Weissella* strains is yet to be known.

Further adhesion ability of *W. paramesenteroides* was evaluated by in vitro mucin adhesion. Three strains FX1, FX2, and FX5 showed up to 75% relative adhesion to mucin. The in vitro mucin adhesion studies of *W. paramesenteroides* type strain ATCC33313 reported by Ku et al. was slightly lower (only up to 50%) [26]. Collectively, these studies related to adhesion such as CSH measured by SAT and autoaggregation assay, bile-induced biofilm formation, and in vitro mucin adhesion ability describe the adhesion potential of *W. paramesenteroides* strains.

Weissella has received a considerable attention as potential probiotic organism which suggests the necessity to evaluate the safety aspects of these strains as per the recommendations of FAO/WHO (2002) [36]. Any probiotic strain intended for the human consumption should not carry genes that render antibiotics resistance due to their associated risk involved in horizontal transfer of such genes from probiotics to the opportunistic pathogens [37]. In the present study, isolated strains did not show resistance to the studied antibiotics except vancomycin, which is a common characteristic for all the known lactobacilli. Similar results were also reported for *W. cibaria* and *W. confusa* strains isolated from Kimchi, a Korean-fermented vegetable food [38]. None of our *Weissella* strains showed hemolytic reaction or DNase activity which is an associated characteristic of pathogenic virulence and neither have they produced tyramine from tyrosine which causes toxicological effects upon its accumulation in large quantities [39]. More detailed safety tests including in vivo studies must be done to establish the safety of these strains for animal consumption.

Synergistic synbiotics are combination of probiotics, selected based on specific beneficial effects on the host, and prebiotics, selected to specifically stimulate the growth and/or activity of the particular probiotic organisms. Here, prebiotics may also increase the levels of the beneficial host GI microbiota, but the primary target is to increase the biomass of the ingested probiotic organism [11]. Therefore, to archive the benefits of synbiotics, it is noteworthy to explore prebiotic utilization potential of the *W. paramesenteroides*. Five tested *Weissella* strains showed varying growth, pH drop, and prebiotic scores in the presence of different prebiotics, viz., FOS, GOS, and XOS. Strains showed preference for the utilization of GOS and FOS but did not utilize XOS well. However, minimal growth on XOS with no prominent pH drop may be due to the small amounts of xylose and arabinose present as impurities [15]. To the best of our knowledge, this is the first study on GOS and FOS utilization potential of *W. paramesenteroides*. GOS utilization potential of *W. paramesenteroides* strains was further explored by evaluating β-galactosidase activity, since galactose containing oligosaccharides can be catabolized by the glycosyl hydrolases, such as β-galactosidase enzyme [10]. In the present study, *W. paramesenteroides* produced tenfold higher β-galactosidase activity when grown in the presence of GOS compared to glucose. Similar result of higher β-galactosidase activity by *Weissella* strains in the presence of lactose than glucose was reported by Lee et al. [40], but to the best of our knowledge, *Weissella* has not been studied for β-galactosidase activity in the presence of GOS. However, there are previous reports on lactobacilli and bifidobacteria strains suggesting the genes encoding β-galactosidases are upregulated when grown in the presence of GOS [21, 41]. Herein similarly *W. paramesenteroides* strains showed induced β-galactosidase activity in the presence of GOS, further providing evidence for positive correlation between GOS utilization potential exhibited by these strains and their enhanced β-galactosidase activity.

Another important functional characteristic feature of a potential probiotic strain is to exert AMA through which they prevent various infections while helping in the homeostasis of gut microbiota, which is principally attributed to extracellular antibacterial metabolites, such as organic acids, antimicrobial peptides, and hydrogen peroxide [3]. *Weissella* strains FX1, FX5, and FX9 exhibited higher growth and pH drop in the presence of FOS compared to GOS. Similarly, higher AMA against *E. coli* was found in the presence of FOS compared to GOS. Lower AMA of EPC extracted from FX5 against *E. coli* and *Staph. aureus*, further provides an evidence that the AMA could be mainly due to the produced organic acids. Therefore, AMA of CFS obtained after the growth of these *Weissella* strains in the presence of prebiotics could provide an evidence for the effective utilization of the prebiotics and thus their growth potential on prebiotic sources suggesting synbiotic potential of these strains. Based on the obtained results, FX5 and FX9 were selected to evaluate residual prebiotics and SCFAs production in CFSs after growth in the presence of prebiotics by TLC and HPLC, respectively.

TLC analysis of CFSs of *Weissella* strains FX5 and FX9 grown in the presence of GOS or FOS depicted their preferential utilization of GOS and FOS, respectively. Moreover, the residual GOS or FOS analysis on TLC of these strains confirmed their preferential low molecular weight GOS or FOS utilization, as only upper low molecular weight spots were disappeared, while residual high molecular weight FOS or GOS was observed in the CFS till the end of fermentation. Similar result of unutilized high molecular weight FOS by bifidobacteria on TLC was reported earlier by Perrin et al. [42]. Moreover, TLC analysis of GOS showed increased intensity of galactose spots in the CFS compared to control GOS, describing the possible accumulation of galactose in the CFS after utilization of GOS. The reason behind it could be the higher rate of galactose production from catabolism of GOS by enzymatic activity over the rate of galactose uptake or intracellular metabolism of galactose [43]. Collectively, the

enhanced β-galactosidase activity with GOS and appearance of galactose accumulation in the CFS on TLC provided an evidence for the GOS utilization potential of these two strains.

End products of prebiotics fermentation by probiotic bacteria of *Lactobacillaceae* family are mostly lactic acid and SCFAs, namely, acetic acid, butyric acid, formic acid, and propionic acid, which are subsequently used by the host as a source of energy and as barrier for the pathogenic microbes [44]. In the present study, both the strains FX5 and FX9 showed increased production of total SCFAs with FOS, and these results can be comparable with the more AMA against *E. coli* and *Staph. aureus* of the FOS grown CFSs of these strains than with GOS grown CFSs. Increased production of 2C containing acetic acid and 3C containing propionic acid in the presence of prebiotics indicates the metabolic complexity of these isolates, which might be due to their differences in the activated metabolic pathways or gene regulatory networks. However, to rationalize the preference for GOS or FOS or glucose by these two strains at this stage, with the current available knowledge, is difficult. A thorough experimentation to understand the genetic makeup and metabolic fluxes in the intermediary metabolic pathways is necessary for rationalization of GOS or FOS utilization. At this stage it can be concluded that both the strains produced more total SCFA in the presence of prebiotics.

In conclusion, in the present study, *W. paramesenteroides* strains isolated from fruits were evaluated for probiotic potential and prebiotic utilization ability. The five *W. paramesenteroides* strains are (i) able to survive in low pH and in the presence of ST; (ii) possess important functional properties of food grade bacteria such as biofilm formation, mucin adherence, and autoaggregation; (iii) exhibit prebiotic (FOS and GOS) utilization ability, with additional AMA against *E. coli* and *Staph. aureus*. Higher levels of secreted β-galactosidase in the presence of GOS and galactose accumulation in CFS observed on TLC plates provided evidence for the GOS utilization potential of FX5 and FX9 and substrate-based gene induction mechanism. Further, concomitant decrease in the intensity of residual FOS on TLC profile enhanced acetic acid production, and AMA of these two strains in the presence of FOS against *E. coli* provided evidence for the synbiotic potential of FX5 and FX9. Based on functional characterization, *W. paramesenteroides* strains FX5 and FX9 can be considered as a strong candidate for probiotic applications owing to their viability in low pH and ST, owing to adhesion abilities such as mucin adhesion and bile-induced biofilm formation, and most importantly owing to their synergistic synbiotic properties such as SCFAs production and AMA with GOS and FOS. Additionally, both these strains fulfilled the safety aspects of probiotics, as they do not harbor any virulent trait, such as antibiotic resistance, biogenic amine production, hemolysis, and DNase production. These two strains FX5 and FX9 can be taken forward for the detailed in vivo evaluations for their beneficial synergistic synbiotic effects.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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