Lactobacillus fermentum strains from rice water and lemon pickle with potential probiotic properties and wastewater treatment applications

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Abstract

Enteric infection by faecal contamination of drinking water is a major concern in developing nations contributing to the huge burden of malnutrition and infant mortality. We have developed a holistic approach, named as gut to gutter approach, to treat wastewater treatment, by screening potential probiotic strains from staple food sources. The strains could be potentially used for competing out the enteric pathogens in different applications such as food and wastewater treatment. We focused on two strains isolated from rice water (RS) and lemon pickle (T1), identified as *Lactobacillus fermentum* and characterized for their probiotic and wastewater treatment capabilities. The strains showed many probiotic properties (tolerance to low pH (pH 3), bile salts up to 0.5% and simulated gastric juice at low pH and binding to extracellular matrix molecules, heparin and gelatin. Auto-aggregation of T1 was in the range of 85% and the strain exhibited significant co-aggregating ability with enteric pathogens, *Klebsiella pneumoniae, Salmonella enterica* and *E. coli* (MDR) with 48%, 79% and 65% respectively. Both strains had a higher binding affinity to gelatin and heparin, compared to widely claimed “probiotic” *Bacillus clausii* and the pathogenic *E. coli* ET. The strains had high galactosidase activities. Further, the cell free supernatant from RS showed BLIS (bacteriocin like inhibitory substance) activity against *Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella enterica* at 60%, 48% and 30% respectively. T1 strains and *B. clausii* effectively reduced the coliform count by by 90% (1-log) when immobilized in a biofilter to treat wastewater.

Introduction

Fermented foods and drinks have been the most explored source for isolation of beneficial (probiotic) bacteria effective in controlling infections of the gut (Kanauchi *et al.* 2018). Probiotic bacteria are defined as live microorganisms that exert beneficial effects on human health when ingested in sufficient quantities (Oh *et al.* 2018b). These effects improve intestinal microbial balance, immunomodulation, promote healthy gut microflora and inhibit the proliferation of harmful bacteria in the gastrointestinal barrier (Repa *et al.* 2015; Xue *et al.* 2017), and offer new dietary alternatives for the stabilization of the intestinal microflora (Spanhaak, Havenaar and Schaafsma 1998; Bansal *et al.* 2013).

Despite huge investment in development of probiotics as therapeutics/drugs, so far very few of them, if any, have succeeded in full scale clinical trials and are approved as drugs. The reason is being the complexity of gut microbiome dynamics as a function of host, food and other complicated environmental factors. We feel there needs to be a more precise understanding of the gut microbiome to be altered by probiotic and prebiotic applications. Moreover, there are large regulatory barriers for any drug approval for human therapeutics. There is a huge scope of using the same probiotics strains for other applications where regulatory hurdles are less such as wastewater treatment for removal of faecal contamination. These applications have potential to reduce the overall cost of probiotic development. Hence we attempted the same putative probiotics strains for wastewater treatment. We made biofilters immobilizing with different strains and tested their capacity to remove total coliforms in wastewater. Biological treatment of wastewater by protective culture based microbial consortium in biofilters can be
an effective strategy to treat wastewaters. Biofilms with commensal bacteria can significantly reduce pathogen load biodegrading nitrogenous compounds and carbonaceous materials (Galvan et al. 2016).

As per FAO/WHO guidelines, evaluation of probiotics should follow stringent phenotypic and genotypic characterization (Toba et al. 1995; Reid Gadir and Dhir 2019). Fermented rice water and pickle are widely used in traditional food/beverage across the globe. Rice water, a starch suspension retrieved after boiling rice, is widely used to treat diarrhoea induced by gastrointestinal diseases and cholera for decades (Wong 1981; Sheila and Peerce 1992; Gregorio et al. 2009). They are rich sources of beneficial microbes (Giri et al. 2018; Jeygowri et al. 2015). Pickles too when part of the diet in restricted quantities have imparted similar effects (Zokaeifar et al. 2012). Hence we isolated several strains from these sources and did a series of in vitro investigations to evaluate their potential probiotic properties such as host matrix binding, tolerance to low pH, bile salts and gastric juices, anti-pathogenic activities and to connect the gut to gutter associations, few strains were employed as biofilter components in wastewater treatment (Shokryazdan et al. 2014; Khalil et al. 2007; Štyriak et al. 2003).

**Materials And Methods**

**Media, Chemicals and Instrumentation Used**

Lemon pickle was obtained locally (Amritapuri, Kollam, Kerala, India) and rice water was aseptically fermented for 6 hours. De Man, Rogosa and Sharpe agar (MRS agar), and Nutrient Gelatin was obtained from HiMedia, India for isolation of Lactobacillus strains and gelatinase activity respectively. Gelatin was obtained from HiMedia, India and Heparin from Biological E. Limited, Hyderabad, India for matrix binding studies. Chromocult® Coliform Agar was purchased from Merck, India. Bile salts, Pepsin and Ortho-nitrophenyl-β-D-galactopyranoside discs (ONPG) discs were procured from HiMedia, India. Solvents, hexadecane and xylene was procured from Sigma-Aldrich, India. Dialysis tubing (3.5K MWCO) was purchased from Thermo Fischer Scientific, India. Absorbance measurements employed synergy microplate reader using Gen5.2.05 Software. Fluorescent microscope (Olympus IX71) and (Olympus BX51) was employed for matrix binding studies.

**Microbial strains used in the study**

Bacillus clausii spore suspensions (Enterogermina®), Klebsiella pneumoniae (MTCC 3384) from Microbial Type Culture Collection, Chandigarh, India, Pseudomonas aeruginosa wild type strain, PAO1 (ATCC 15692), from American Type Culture Collection, Manassas, VA, USA, Escherichia coli ET (enterotoxigenic), Escherichia coli (MDR), Salmonella enterica and Staphylococcus aureus was gifted by Dr. Bhabatosh Das, THSTI, Faridabad, India.
Isolation of probiotic strains from rice water and lemon pickle

Locally available lemon pickle and fermented rice water were chosen to isolate the *Lactobacillus* strains in MRS agar at 37 °C by spread plate method. The rice water sampled from cooked rice was aseptically fermented for 6 h and serially diluted up to 10^5. In both cases, few randomly selected colonies were further screened on its binding to gelatin. Gelatin coating was performed in flat bottom 96 well plates by incubating 100 µL of 10 mg/mL gelatin in each well at room temperature for 1 h followed by rinsing with phosphate buffer saline (PBS) thrice. The MRS positive colonies suspended in PBS were added to each well and kept at room temperature. After 10 min, the bacterial suspension was discarded and washed with 100 µL PBS 5 times and the fifth wash was spread plated. More stringent similar volume wash was done subsequently with 1 N NaCl in PBS and then 1% dimethyl sulphoxide (DMSO) in PBS. The uniform small pinheaded colonies obtained from 1% DMSO-PBS fifth wash, which is considered as a strong binder to the gelatin, was selected and subcultured for characterization. Rice water isolate was termed RS and pickle isolate as T1 (Kumar and Ghosh 2012).

Genomic DNA isolation and 16SrRNA sequencing for identification of strains

Genomic DNA isolation was performed by phenol chloroform method (Green and Sambrook 2017; Xu *et al.* 2019; Porayath *et al.* 2018). The DNA isolated was subjected to normal PCR with the help of 16s rRNA gene primers forward primer (5'–AGAGTTTGATCCTGGCTCAG–3'), reverse primer (5'–ACGGCTACCTTGTTACGACTT–3') producing an amplicon of length 1.5 kb. After sequencing BLAST analysis was performed (Zhang *et al.* 2000; Salim *et al.* 2019).

Evolutionary analysis

BLASTn analysis of the sequences were done with the default parameters. The results obtained for aligned fasta sequences were downloaded from the NCBI BLAST and phylogenetic analysis was carried out with the help of MEGA-X version 10.0.5. Fasta files were imported and aligned using MUSCLE and the resulting.meg files were used to construct the phylogenetic tree using the Neighbor-Joining method with default parameters (Saitou and Nei 1987; Tamura, Nei and Kumar 2004; Kumar *et al.* 2018).

Assessment of probiotic properties of the strains

Acid tolerance ability
To determine the acid tolerance of the strains, overnight culture of the strains (were inoculated into tubes of MRS broth previously adjusted to pH values (1.5 and 3) using 1N HCl and 1N NaOH. The cultures adjusted to 0.1 OD (in accordance with McFarland Standard) was inoculated and aliquots of cultures exposed to pH 1.5 and 3 at 0 h and 3 h were plated on to MRS agar and viable counts were determined. MRS broth maintained at pH 7 was used as the control (Kim et al. 2019). Experiments were repeated in triplicates and average of 3 independent values were plotted.

**Bile Tolerance ability**

The bile salt tolerance was checked in MRS agar incorporated with bile salts, adding varying concentrations from 0.2% to 2%. Aliquots of overnight cultures was spread plated onto the surface of the bile-salt-containing MRS agar at 0.2% and 0.5% and viable counts were taken after 3 h of exposure. MRS broth without bile salts was used as control (González-Vázquez et al. 2015).

**Tolerance to Simulated Gastric Juice (SGJ):**

The strains were centrifuged and resuspended in saline equivalent to an absorbance value of 1 at 600 nm. The cultures were then inoculated to simulated gastric juice (125 mM NaCl, 17 mM KCl, 45 mM NaHCO₃, 3 g Pepsin) adjusted to 3 different pH ranges 2, 3 and 7. Absorbance values after exposure to 6 h was measured at 600 nm (Hassanzadazar et al. 2012).

**Elucidation of cell surface properties**

**Microbial Adhesion to Hydrocarbon Test (MATH) assay for hydrophobicity**

Bacterial cell surface hydrophobicity was assessed by measuring adhesion to hydrocarbons, hexadecane and xylene. Overnight cultures of RS and T1 were centrifuged at 9,000 g for 10 min at 4 °C. The pellet was washed with phosphate urea magnesium buffer (PUM buffer- 22.3 g K₂HPO₄, 7.26 g KH₂PO₄, 1.80 g urea, 0.2 g MgSO₄·H₂O, pH 7.1). Absorbance at 600 nm was adjusted to 1 OD. To 5 mL cell suspension 1 mL hexadecane was added. The two-phase system was vortexed for 2 min followed by incubation at 37 °C for 1 h. Suspension was vortexed for 3 min and then incubated at room temperature for 1 h. Aqueous phase was removed, and absorbance was measured at 600 nm. Similarly, a two-phase system with xylene was performed. The percentage of cell surface hydrophobicity was expressed as (H %) = (1-A1 /A0) ×100 where A1 represents the absorbance of aqueous phase after 1 h and A0, at time t = 0. *Bacillus clausii*, a known probiotic organism was used as the positive control (Xu et al. 2019).
Autoaggregation assay

The strains (RS and T1) were grown in MRS broth and incubated at 37 °C. The overnight cultures were centrifuged (5,000 g, 15 mins, 4 °C), harvested cells washed twice with phosphate buffer saline (PBS) and resuspended in the same to 4 mL calibrated to an OD of 0.2 (10^8 CFU/mL). The cells were vortexed for 10 s and the autoaggregation was determined at 3, 5 and 24 h respectively. The top portion of the suspension was transferred to another tube with 3.9 mL of PBS and the absorbance (A600) was measured each hour. The autoaggregation percentage is expressed as: Autoaggregation (%) = \( \frac{1 - A_t}{A_0} \times 100 \) Where At denotes the absorbance at time t = 1-5 h and 24 h and A0 the absorbance at t = 0. *Bacillus clausii*, a known probiotic organism was used as the positive control (Sorroche *et al.* 2012; Ben Taheur *et al.* 2016).

Coaggregation assay of probiotic strains with pathogenic strains

Coaggregation of probiotic strains with pathogens was investigated. The bacterial cell suspension was resuspended in PBS to approximately 10^8 CFU/mL, and 2 mL of each bacterial suspension was mixed with 2 mL of RS and T1 each and vortexed for 10 s. (*Salmonella enterica*, *E. coli* MDR, *Klebsiella pneumoniae*). Control tubes, each with 4 mL of bacterial suspension alone, were maintained at room temperature. The degree of coaggregation was measured at 600 nm for 3 h, 5 h and 24 h respectively (Collado, Meriluoto and Salminen 2008).

Bacterial attachment to immobilized extracellular matrix (ECM) proteins

Gelatin and heparin (1 mg/mL, 50 μL) was added to 96 well plates and was incubated at room temperature for 1 h. Unbound proteins were removed by washing with PBS. Absorbance of the strains was adjusted to 0.1 (600 nm) and 50 μL of each of the bacterial suspension was transferred to the coated plates and incubated for 1 h at room temperature. The wells were washed with 1X PBS for 3 times, 50 μL of formalin was added and the plate was kept undisturbed for 20 min to fix the bacteria and was washed with PBS. Crystal violet (0.1 %, 50 μL) stain was added for 1 minute. Excess stain was removed by washing and the plates were dried after which 50 μL of acetic acid was added into the wells to solubilize the dye. Absorbance was measured at 595 nm in Synergy microplate reader using Gen5.2.05 Software. *Bacillus clausii* and *E. coli* ET strain were used as controls (Yadav *et al.* 2014; Nishiyama *et al.* 2015).

Microscopic examination of binding efficiency of the strains (fluorescent staining and crystal violet staining)
Microscopic observation of the probiotic binding to ECM was performed with some modifications (Shahara et al. 2012). Cultures (RS and T1 in MRS broth) adjusted to 0.1 OD were inoculated into sterile coverslips placed in 6 well microtitre plates and were kept for incubation for 5 h. The wells were washed with 1 X PBS to remove planktonic cells and coverslips transferred to glass slides were heat fixed at 55 °C for 20 min. Slides were then kept overnight at 4 °C and air dried (15 min). Fixed slides were stained with 0.02% acridine orange for three min in the dark, washed with distilled water and then air dried at room temperature for 15 min. The attached cells were then imaged using a fluorescent microscope (Olympus IX71). Another set of matrix bound coverslips were stained with crystal violet (0.1%) and observed under 100 X magnification (Olympus BX51).

Biofilm formation of probiotic strains

In order to perform biofilm quantification, 2 mL of overnight culture of RS and T1 were inoculated into 6 well microtitre plates, with an initial turbidity of 0.25 OD at 600 nm. MRS broth was employed as control. Plates were then left in a static condition for 48 h at 30 °C. Biofilm formation was analysed by standard crystal violet assay as described by Gómez et al. Quantification was made based on the equations, non-biofilm producers [OD ≤ ODC], weak biofilm producers [ODC < OD ≤ 2 x ODC], moderate biofilm producers [2 x ODC < OD ≤ 4 x ODC], strong biofilm producers [4 x ODC < OD] where ODC (cut-off) is mean OD value of control. B. clausii was kept as a positive control. Visualization of biofilm formation was further performed using acridine orange and crystal violet staining (Gómez et al. 2016).

Detection of β -galactosidase activity:

Qualitative determination of the rate of lactose fermentation was done by β-galactosidase assay. A single colony of RS and T1 was applied to Ortho-nitro phenyl β –D-galactopyranoside discs (ONPG) in MIC tubes followed by addition of 100 µL of saline. Reduction of ONPG to ONP (ortho nitrophenol) was indicated by a color change to yellow at room temperature (Gómez et al. 2016; Cebeci and Gürakan 2003).

Coliform reduction in sewage aided with probiotic biofilters

The efficiency of the probiotic strains, T1 and Bacillus clausii for their ability to form biofilms embedded in biofilters was utilized to reduce coliform count in sewage. Pseudomonas aeruginosa (PA01) was used as a positive control. 1 L bottle employed as filter was filled with pre washed and dried sand, charcoal, coarse gravel, fine gravel, big gravel in the ratio of 3:3:1:1:1 respectively to a total bed height of 18 cm. The filter was aseptically washed for 30 min, followed by inoculation of overnight culture of T1, Bacillus clausii and Pseudomonas aeruginosa at OD (600) of 0.3 into the respective filters for 72 h. A control filter was maintained devoid of biofilm. After 3 d, the biofilter was washed with 0.9% percent saline without disturbing the biofilm and sewage was introduced at a flow rate of 35 mL/min (slow sand
filter linear velocity: 0.3 m/h). The reduction in fecal coliform (*Escherichia coli*) was checked in Chromocult® Coliform Agar (Verschuere *et al.* 2000; Prol-García and Pintado 2013).

### Detection of Gelatinase activity

Gelatinase production of the strains was checked by spotting 1µL aliquots of the 24 h cultures on to the surface of nutrient gelatin plates (HIMEDIA). Plates were incubated for different time period and temperature, 37 °C, 42 °C (48 h), 25 °C (72 h) and 10 °C and 15 °C (10 d). After incubation, the plates were maintained at 4 °C for 2 h and gelatin hydrolysis was recorded as opaque halos around the colonies (Oh *et al.* 2018).

### Bacteriocin extraction by pH mediated adsorption desorption method and activity assay:

Initially, heating the culture broth to 60 °C was done in order to prevent the inactivation of bacteriocin by proteases present in the culture medium. Adsorption of bacteriocin to producer cells was facilitated by adjusting the pH to 6.0 using 1M NaOH followed by steering it for 30 min at 4 °C. The cells were harvested by centrifuging at 10,000 g for 25 min at 4 °C and pellets were washed twice with sterile 0.1 M phosphate buffer (pH 6.5). Pellets resuspended in 100 mM NaCl, was adjusted to pH 2.0 (1N HCl), stirred for 12 h at 4 °C. Centrifugation of cell suspension at 10,000 g for 25 min was done and supernatant dialyzed against distilled water at 4 °C for 24 h (dialysis tubing 3.5 K MWCO, Thermo Fischer Scientific). The protein concentration was determined by Bradford assay (Elegado, Kim and Kwon 1997; Zhang *et al.* 2009; Zhang *et al.* 2013). The dialyzed samples were tested against indicator pathogens *S. typhi*, *S. aureus* and *K. pnuemoniae* by microtitre inhibition assay (Vijayakumar and Muriana 2015).

### Statistical analysis

Statistical analysis of data obtained was performed by conducting Two-way RM ANOVA and values were expressed as mean ± SD (Standard deviation of the mean) values of 3 independent experiments using the software Graph Pad Prism 6. Significance levels were at *P* ≤ 0.05,**P* ≤ 0.01,***P* ≤ 0.001 and ****P* ≤ 0.0001.

### Results

**Isolation of probiotic strains, genomic DNA isolation and 16S rRNA typing**

The rice water isolate (RS) and pickle isolate (T1) was identified as *Lactobacillus fermentum* strains based on their molecular characterization. The strains were deposited in GenBank under accession no: (i)
MN410703 (rice water isolate) and (ii) MN410702 (pickle isolate) respectively.

**Evolutionary analysis**

The strain MN410703 (RS) was not closely related to any other strains as analyzed from the BLAST analysis which makes it an interesting candidate to elucidate the complete genome sequence. (Fig.1a). The strain MN410702 (T1) demonstrated an evolutionary relationship with the strain MK639007 (Fig.1b).

**Assessment of probiotic properties of the strains**

**Acid tolerance ability**

When compared to the control (pH 7) after exposure for 3 h, the colony counts of RS did not decrease in both pH 1.5 and 3 indicating their ability to persist and grow in the high acidic environment ($P \leq 0.01$). (Fig. 2a) However, there was a negligible reduction in growth when the organism is exposed to pH 1.5 for 3 h. T1 after 3 hours of exposure demonstrated increased growth at pH 3 compared to the control (pH 7) (Fig. 2b).

**Bile Tolerance ability**

Viable counts of T1 and RS were plated after 3 h of exposure to 0.2 and 0.5% of bile salts. Results clearly demonstrated that both RS and T1 significantly increased in counts by one log when exposed to bile salt concentrations of 0.2% ($P \leq 0.01$) when compared with the control. T1 could tolerate 0.5% of bile salts which was evident by the increase in CFU/mL. (Fig. 2c).

**Tolerance to Simulated Gastric Juice**

The absorbance values after 6 h of exposure clearly demonstrated that with increasing time, T1 showed increase in absorbance in simulated gastric juice (SGJ) at pH 2 and pH 3 ($P \leq 0.001$) (Fig. 2d) while RS showed increased in absorbance at pH 3 compared to the control pH 7 ($P \leq 0.01$) confirming the ability of the strains to tolerate the high acidity in the gastric environment (Fig. 2e).

**Elucidation of cell surface properties**

**Microbial Adhesion to Hydrocarbon Test (MATH) assay for hydrophobicity**
The result indicated that compared to *Bacillus clausii*, RS and T1 had more affinity towards xylene. \((p \leq 0.01\) in ranges of 88% and 90% for RS and T1 respectively (Fig. 3a). However, affinity to hexadecane was in the ranges of 25% and 21%. The results confirmed the ability of strains to bind to hydrophobic barriers within the gut epithelium.

**Autoaggregation assay**

Aggregation properties of the strains were compared with *B. clausii* and *E. coli* ET. After 5 h compared to *Bacillus clausii*, T1 exhibited higher percentage of autoaggregation in the range of 85% \((P \leq 0.001)\). After 24 h of incubation both strains exhibited 85% of autoaggregation when compared with the control (Fig. 3b). This property will essentially help the probiotic strains to adhere to the gut epithelium and enhance their coaggregation abilities.

**Coaggregation assay**

In agreement with the autoaggregation results, T1 coaggregated with *Salmonella* spp in a range of 78% within 5 h of incubation. While with *E. coli* and *Klebsiella* spp. percentages were 65 and 45% respectively after 24 h of incubation \((P \leq 0.0001)\) (Fig. 3c). RS coaggregated with all the strains in the range of 65% after 24 h \((P \leq 0.0001)\) (Fig. 3d). High coaggregation percentages reveal the ability of the strains under investigation to competitively exclude pathogens from the gut epithelia thereby activating an immune response.

**Bacterial attachment to immobilized extracellular matrix (ECM) proteins**

To establish the strains ability to bind to the ECM proteins, *in vitro* assays were performed followed by microscopic observation. T1 demonstrated increased binding efficiency to gelatin matrix until 5 h consistently when compared to *B. clausii*, \((P \leq 0.001)\). Binding of RS to gelatin matrix was similar to the pathogen model (*E. coli* ET after 5 h. (Fig. 4a). With heparin, RS and T1 bound to the matrix with similar affinity as demonstrated as *B. clausii* (Fig. 4b). It was observed that binding of *E. coli* was significantly reduced after 5 h of incubation. Results were confirmed by fluorescent microscopy and crystal violet staining (Fig. 5). The strains were found to be binding abundantly to the matrix after 5 hours of incubation with gelatin (Fig. 5a-h) and heparin (Fig. 5i-p) when compared to *B. clausii* and *E. coli* ET as revealed by microscopic assay.

**Biofilm formation**
RS and T1 were found to be moderate biofilm producers indicated by the absorbance values obtained when compared with *B. clausii* (Fig. 6a). Further validation of the observation was confirmed by CV staining and fluorescent microscopic observation (acridine orange) of the biofilms. Both RS (Fig. 6b-c) and T1 (Fig. 6d-e) formed biofilms abundantly on the surface indicated by the microscopy which could strongly correlate with their aggregation, co-aggregation abilities and hydrophobicity percentages.

**Detection of β-galactosidase activity:**

β-galactosidase production of RS and T1 was confirmed qualitatively with ONPG discs and within 24 h yellow colour development in MIC tubes indicated that the strains were early lactose fermenters (Fig. 6f).

**Coliform reduction in sewage aided with probiotic biofilters**

The *E. coli* count (blue colonies in the Chromocult® Coliform Agar) was found to be reduced by 1 log (90%) when sewage was passed through the biofilters augmented with T1 and *Bacillus clausii* biofilms, compared to the raw sewage (*P* ≤0.001). It was quite evident from the observation that biofilter devoid of probiotic biofilms was less effective in reducing *E. coli* counts (Fig. 7). This confirmed the prospective application of these strains in biofilter embedded reduction of coliforms.

**Detection of Gelatinase activity**

Both strains showed no gelatin hydrolysis zones after different incubation conditions indicating the absence of gelatinase which is a virulence trait (Table 1).

**Table 1 Gelatinase activity of RS and T1**

| Temperature and time of incubation | Gelatinase activity of the strains | RS | T1 |
|-----------------------------------|-----------------------------------|----|----|
| 37 °C (48 h.)                     | -                                 | -  | -  |
| 42 °C (48 h.)                     | -                                 | -  | -  |
| 25 °C (72 h.)                     | -                                 | -  | -  |
| 10 °C (10 d)                      | -                                 | -  | -  |

Gelatinase activity of RS and T1 at different temperature and time of incubation. (-) sign indicates the absence of gelatinase activity.
Bacteriocin extraction by pH mediated adsorption desorption method and activity assay

RS specifically demonstrated inhibitory activity against the pathogens *S. enterica*, *S. aureus* and *K. pneumoniae* (MTCC 3384) with percentage inhibition rates of 30%, 48% and 60% respectively (*P* ≤ 0.001) (Fig. 6f). However, the effect of T1 was not prominent against the strains (Fig. 8).

Discussion

The prolonged use and synthesis of antibiotics in multiple communities and hospitals has fuelled the crisis of antimicrobial resistance even more (Chandler 2019). Recent studies implicate that over 70% of global antimicrobial applications are accounted by animal food sector (Van Boeckel et al. 2019). Sewage treatment plants are becoming the breeding ground of AMR genes, where sensitive strains are freely mixing with resistant ones derived largely from non-therapeutic sources particularly from food and agriculture sources (Pazda *et al.* 2019). It is high time we develop an integrated antimicrobial use policy emphasizing to manage this health emergency. Moreover, development of probiotics to alter microbial dynamics to favourable microbiome is complex, as probiotic engineering by strains need a more nuanced approach by analysing the microbiome composition and the keystone species in that particular environment. Hence it is important we look for approaches which can reduce the research and development (R&D) cost of this approach by exploring wide number of applications for the putative probiotic strains. Even though metagenomic and whole genome analysis give us some clues about the composition of the microbiome, their dynamics is very difficult if not impossible for predicting the keystone species. We are developing different bacteriophages against enteric pathogens, which can bind to host cell matrix molecules such as gelatin, fibronectin, heparin (Porayath *et al.*, 2018) and also trying to understand the role of knocking out these pathogens on overall microbiome dynamics by 16s ribotype based microbiome analysis with next generation sequencing technologies (unpublished results). In parallel, we need traditional culture-based approach and trial and error methods for which one or few species need to be experimentally tested for their potential to reduce the target pathogens in complex ecosystems such as food or wastewater.

Antimicrobial resistance (AMR) gene abundance is directly related to societal, health and environmental factors in different countries (Hendriksen *et al.* 2019). Several initiatives have been introduced to address this growing concern among which probiotics play a major role (Vuotto, Longo and Donelli 2014; Granato *et al.* 2010). Probiotic mediated therapy even though has hazzles to meet in terms of drug trials, application of these in waste water is a less explored approach. Previous reports have suggested that rice bran-based prebiotics in synergy with probiotics in the gut may promote gut health by production of metabolites (Zubaidah *et al.* 2012). Fewer studies have investigated the potential probiotic strains from rice water fermented for a period of 6 to 7 h. Several studies have claimed microbial diversity in different ethnic rice based fermented foods, but not in rice water which is widely drunk across rice cultivating regions in the world, for its potential health benefits (Ray *et al.* 2016). Rice water with proper
supplementation is also recognised as equivalent or better than WHO formulated glucose-based ORS in many respects as described by (Gregorio et al. 2009). Very often this boiled rice water gets fermented while storing. Understanding the possible microbes involved in this fermentation and their effects on human health is very important from a public health perspective, keeping in view of its wide usage in food and wastewater treatment applications.

However, application of these strains in food sector demands identifying bacteria to the species level and validating their probiotic properties and safety guidelines. With stringent regulations from FAO and supported by the World Health Organization, the definition, purpose and applications of probiotics are under scrutiny and demand consistency in investigations of properties to be validated for food applications (Fao et al. 2002).

An effective prerequisite property of probiotic strains is their ability to survive the harsh conditions in the gastrointestinal tract (Mantzourani et al. 2019). Therefore, the two strains namely RS and T1 identified as L. fermentum MN410703 and MN410702 respectively were tested for their ability to tolerate the acidic condition, bile salts and simulated gastric juice conditions. At pH 2, organisms were able to survive, but they grow better at pH 4 similar to observations made by (Honey Chandran and Keerthi 2018). In contrary viable counts indicated that strains were able to tolerate low pH ranges of 1.5 and 3. This is in agreement with studies done in probiotic strains isolated from cocoa fermentations by (Lacerda et al. 2013). The resistance to low pH is an important characteristic for the food industry as they withstand acidic environments for long periods.

Bile tolerance is crucial for growth and survival of the strains in the proximal part of the small intestine. The liver synthesizes bile salts from cholesterol and is an essential candidate in absorption and digestion of fats (Meira et al. 2012). Staying time of food in the small intestine is 4 to 6 h and the mean bile concentration of bile is 0.5% in the small intestine. Earlier investigations have proved that viability in 0.3% is considered to be optimum for bile resistant strains as per (Gilliland, Staley and Bush 1984). Our strains showed good tolerance after 6 h of exposure to 0.2% and 0.5% concentrations. The acidic gastric condition in the stomach destroys most of the microorganisms. RS and T1 survived in simulated gastric juice for 6 h and showed significant growth. Their significant transit tolerance were in full agreement with similar tolerance of probiotic strains isolated from broiler chicken (Nallala, Sadishkumar and Jeevaratnam 2017; Reuben et al. 2019).

Hydrophobicity, an important factor of a bacterial cell that shows the adhesive reaction to the intestinal surface, also enhances their tendency to form biofilm (Krasowska and Sigler 2014; Savage 1992). The investigations proved that our strains showed affinity to xylene in higher ranges when compared to hexadecane. Strains with hydrophobicity more than 40% shows they are hydrophobic (Sidira et al. 2015) and effectively colonize the intestinal walls. Bacterial cell surface hydrophobicity and autoaggregation ability are directly correlated (Tuo et al. 2013). Autoaggregation can prevent or act as a barrier against the colonization of pathogens (Kos et al. 2003). Coaggregation is related to the ability to interact closely with other bacteria(Chen et al. 2019). Strong aggregating nature of the probiotic strain may help to achieve an
adequate mass to form biofilms (Zivkovic et al. 2015). In this study all the strains showed good aggregation property. T1 showed a consistent range of autoaggregation properties until 24 h. They were also strongly coaggregating with pathogens indicating their competitive exclusion properties. Probiotic strains can be used as an alternative approach to reduce the inhibitory effects of pathogenic biofilm formation by food borne pathogens (Berrios et al. 2018). In alignment with aggregation properties, all the strains were found to be moderate biofilm producers. Correlation between autoaggregation, coaggregation and biofilm formation properties of the strains were in agreement with the observations by (Vlková et al. 2008):

Higher coaggregation and biofilm formation of T1 and B. clausii strains were a positive cue for application of these strains in biofilms to reduce the coliform count in sewage which impacted as a multi composite application of our strains. This would be more exploitable since few investigations have been made using probiotic consortiums for sewage coliform reduction. Production of β-galactosidase an, industrially important enzyme is a characteristic of lactobacillus strains. Addition of lactobacilli producing β-galactosidase as probiotic in milk and cheese help alleviate lactose intolerance symptoms (De Cesare et al. 2017). The absence of gelatinase, metalloproteinases (MMPs) secreted by pathogenic bacteria, provided evidence to the non-virulent nature of the strains under investigation which increases their modulation to be employed in waste water treatment as they are not imparting virulence genes to sewage (Sharma, Sharma and Sharma 2017). Further confirmations should be done to rule out the absence of the virulence factors as per (Popovic et al. 2018). RS based partially purified bacteriocins showed potent antagonistic activity against the target pathogens while, T1 the observations were not much fruitful. This could be explained by the poor antagonistic potential of gram positive bacteriocins in inhibiting few strains of gram-negative genera as explained elsewhere (Hegarty et al. 2016). They may also be of a molecular weight less than 3.5 kDa which would be lost during purification. Further investigations need to carried out in detecting the exact nature of these peptides.

To conclude, our studies are among the first to establish an L. fermentum strain from fermented rice water as potential probiotic with food and sanitation applications, in particular as a novel approach of treating gut enteric pathogens from both wastewater and food/drinks (gut to gutter approach). We could also isolate a potential L. fermentum from lemon pickle which would propagate similiar observations. The need of the hour is to have an better understanding of the complex interaction of the gut microbiome with the putative probiotic strains in space and time. This is particularly relevant to the tropical countries like India, where number of food derived established probiotics is comparatively limited, even though the need to counter enteric infection because of poor sanitation and higher infection rate in tropical climate is very high compared to temperate climate.

Declarations

Ethics approval and consent to participate

Not applicable.
Consent for publication
Not applicable.

Availability of data and materials
The data for the findings are listed here.

Competing Interest
The authors declare that they have no competing interests

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Authors’ contributions
VP, AP and PB designed experiments and wrote the manuscript; SP and BN directed experiments; VP, AP, AJ, SS, MP, AS, RR and PS performed experiments. All authors read and approved the final manuscript.

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References
1. Bansal S, Singh A, Mangal M, Sharma S K (2013) Isolation and characterization of lactic acid bacteria from fermented foods. Vegetos 26 (2):325–330.
2. Ben Taheur F, Koudhi B, Fdhila K, Elabed H , Slama RB , Mahdouani K, Bakhrouf A, Chaieb K (2016) Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens. Microb Pathog 97:213–220.
3. Berríos P, Fuentes JA, Salas D, Carreño A, Aldea P, Fernández F, Trombert A N (2018) Inhibitory effect of biofilm-forming *Lactobacillus kunkeei* strains against virulent *Pseudomonas aeruginosa* in vitro and in honeycomb moth (*Galleria mellonella*) infection model. Benef Microbes 9(2):257-268.

4. Cebeci A, Güürakan C (2003) Properties of potential probiotic *Lactobacillus plantarum* strains. Food Microbiol 20(5):511–518.

5. Chandler CIR (2019) Current accounts of antimicrobial resistance: stabilisation, individualisation and antibiotics as infrastructure. Palgrave Commun 5:53.

6. Chen CC, Lai CC, Huang HL, Huang WY, Toh HS, Weng TC, Chuang YC, Lu YC, Tang HJ (2019) Antimicrobial activity of lactobacillus species against carbapenem-resistant enterobacteriaceae. Front Microbiol 10:789.

7. Collado MC, Meriluoto J, Salminen S (2008) Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 226(5):1065–1073.

8. De Cesare A, Sirri F, Manfreda G, Moniacci P, Giardini A, Zampiga M, Meluzzi A (2017) Effect of dietary supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) on caecum microbiota and productive performance in broiler chickens. PLoS One 12(5):1–21.

9. Elegado FB, Kim WJ, Kwon DY (1997) Rapid purification, partial characterization, and antimicrobial spectrum of the bacteriocin, Pediocin AcM, from *Pediococcus acidilactici* M. Int J Food Microbiol 37(1):1–11.

10. Fao J, Working WHO, Report G (2002) Guidelines for the Evaluation of Probiotics in Food 1–11.

11. Gilliland SE, Staley TE, Bush LJ (1984) Importance of Bile Tolerance of *Lactobacillus acidophilus* Used as a Dietary Adjunct. J Dairy Sci 67(12):3045–3051.

12. Giri SS, Sen SS, Saha S, Sukumaran V, Park SC (2018) Use of a potential probiotic, *Lactobacillus plantarum* L7, for the preparation of a rice-based fermented beverage. Front Microbiol 9:473.

13. Gómez NC, Ramiro JMP, Quecan BXV, Franco BDGdM (2016) Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes, Salmonella Typhimurium*, and *Escherichia coli* O157: H7 biofilms formation. Front Microbiol 7:863.

14. González-Vázquez R, Azaola-Espinosa A, Mayorga-Reyes L, Reyes-Nava LA, Shah NP, Rivera-Espinoza Y (2015) Isolation, Identification and Partial Characterization of a *Lactobacillus casei* Strain with Bile Salt Hydrolase Activity from Pulque. Probiotics Antimicrob Proteins 7(4):242-248.

15. Granato D, Branco GF, Cruz AG, Faria JdAF, Shah NP (2010) Probiotic dairy products as functional foods. Compr Rev Food Sci Food Saf 9(5):455-470.

16. Green MR, Sambrook J (2017) Isolation of high-molecular-weight DNA using organic solvents. Cold Spring Harb Protoc 2017(4):356–359.

17. Gregorio GV, Gonzales ML, Dans LF, Martinez EG (2009) Polymer-based oral rehydration solution for treating acute watery diarrhoea. Cochrane Database Syst Rev. 2:CD006519.

18. Hassanzadazar H, Ehsani A, Mardani K, Hesari J (2012) Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Vet Res forum an Int Q J 3(3):181–
185.
19. Hegarty JW, Guinane CM, Ross RP, Hill C, Cotter PD (2016) Bacteriocin production: a relatively unharnessed probiotic trait? F1000Research 5:2587.
20. Hendriksen RS, Munk P, Njage P, Van Bunnik B, McNally L, Lukjancenko O, Roder T, Nieuwenhuijse D, Pedersen SK, Kjeldgaard J, Kaas RS, Clausen PTLC, Vogt JK, Leekitcharoenphon P, Schans MGM, Zuidema T, Husman AmdR, Rasmussen S, Petersen B, Amid C, Cochrane G, Sicheritz-Ponten T, Schmitt H, Alvarez JRM, Aidara-Kane A, Pamp SJ, Lund O, Hald T, Woolhouse M, Koopmans M P, Petersen HVTN, Aarestrup FM (2019) Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun.10 (1124).
21. Honey Chandran C, Keerthi TR (2018) Probiotic potency of Lactobacillus plantarum KX519413 and KX519414 isolated from honey bee gut. FEMS Microbiol Lett 365(4):1-8.
22. Jeygowri N, Parahitiyawa N, Jeyatilake S, Ranadheera S, Madhujith T (2015) Study on isolation of potentially probiotic Lactobacillus species from fermented rice. Trop Agric Res 26(3): 428-440.
23. Kanauchi O, Andoh A, AbuBakar S, Yamamoto N (2018) Probiotics and Paraprobiotics in Viral Infection: Clinical Application and Effects on the Innate and Acquired Immune Systems. Curr Pharm Des 24(6):710-717.
24. Khalil R, El-halafawy K, Mahrous H, Kamaly K, Frank J, El Soda M (2007) Evaluation of the probiotic potential of lactic acid bacteria isolated from faeces of breast-fed infants in Egypt. Afr. J. Biotechnol. 6(7): 939-949.
25. Kim JA, Bayo J, Cha J, Choi YJ, Jung MY, Kim DH (2019) Investigating the probiotic characteristics of four microbial strains with potential application in feed industry. PLoS One 14:1–16.
26. Kos B, Šušković J, Vuković S. Šimpraga M, Frece J, Matošić S (2003) Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. J Appl Microbiol 94:981–987.
27. Krasowska A, Sigler K (2014) How microorganisms use hydrophobicity and what does this mean for human needs ?. Front Cell Infect Microbiol 4:112.
28. Kumar M, Ghosh M, Ganguli A (2012) Mitogenic response and probiotic characteristics of lactic acid bacteria isolated from indigenously pickled vegetables and fermented beverages. World J Microbiol Biotechnol 28(2):703-711.
29. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549.
30. Lacerda C, Thorsen L, Freitas R, Jespersen L (2013) Strain-specific probiotics properties of Lactobacillus fermentum, Lactobacillus plantarum and Lactobacillus brevis isolates from Brazilian food products. Food Microbiol 36(1):22-29.
31. Mantzourani I, Chondrou P, Bontsidis C, Karolidou K, Terpou A, Alexopoulos A, Bezirtzoglou E, Galanis A, Plessas S (2019) Assessment of the probiotic potential of lactic acid bacteria isolated from kefir grains: evaluation of adhesion and antiproliferative properties in in vitro experimental systems. Ann Microbiol 69:751–763.
32. Meira SMM, Helfer VE, Velho RV, Lopes FC, Brandelli A (2012) Probiotic potential of *Lactobacillus* spp. isolated from Brazilian regional ovine cheese. J Dairy Res 79:119–127.

33. Nallala V, Sadishkumar V, Jeevaratnam K (2017) Molecular characterization of antimicrobial *Lactobacillus* isolates and evaluation of their probiotic characteristics in vitro for use in poultry. Food Biotechnol 31(1): 20–41.

34. Nishiyama K, Nakamata K, Ueno S, Terao A, Aryantini NP, Sujaya N, Fukuda K, Urashima T, Yamamoto Y, Mukai T (2015) Adhesion properties of *Lactobacillus rhamnosus* mucus-binding factor to mucin and extracellular matrix proteins. Biosci Biotechnol Biochem 79(2): 271-279.

35. Oh NS, Joung JY, Lee JY, Kim Y (2018) Probiotic and anti-inflammatory potential of *Lactobacillus rhamnosus* 4B15 and *Lactobacillus gasseri* 4M13 isolated from infant feces. PLoS ONE 13(2): e0192021.

36. Park MR, Ryu S, Maburutse B E, Oh S, Kim S H, O h Sejong, Jeong S Y, Jeong D Y, Oh Sangnam, Kim Y (2018) Probiotic *Lactobacillus fermentum* strain JDFM216 stimulates the longevity and immune response of *Caenorhabditis elegans* through a nuclear hormone receptor. Sci Rep 8:7441.

37. Pazda M, Kumirska J, Stepnowski P, Mulkiewicz E (2019) Antibiotic resistance genes identified in wastewater treatment plant systems–A review. Sci. Total Environ 697:134023.

38. Popovic N, Dinic M, Tolinacki M, Mihajlović S, Vidojević AT, Bojić J, Djokić J, Golić N, Veljović K (2018) New insight into biofilm formation ability, the presence of virulence genes and probiotic potential of *Enterococcus* sp. dairy isolates. Front Microbiol 9:78.

39. Porayath C, Salim A, Palillam Veedu A, Babu P, Nair B, Madhavan A, Pal S (2018) Characterization of the bacteriophages binding to human matrix molecules. Int J Biol Macromol 110:608–15.

40. Prol-García MJ, Pintado J (2013) Effectiveness of Probiotic *Phaeobacter* Bacteria Grown in Biofilters Against *Vibrio anguillarum* Infections in the Rearing of Turbot (*Psetta maxima*) Larvae. Mar Biotechnol 15:726–738.

41. Ray M, Ghosh K, Singh S, Mondal KC (2016) Folk to functional: An explorative overview of rice-based fermented foods and beverages in India. J Ethn Foods 3:5–18.

42. Reid G, Gadir AA, Dhir R (2019) Probiotics: Reiterating what they are and what they are not. Front Microbiol 10:424.

43. Repa A, Thanhaeuser M, Endress D, Weber M, Kreissl A, Binder C, Berger A, Haiden N (2015) Probiotics (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) prevent NEC in VLBW infants fed breast milk but not formula. Pediatr Res 77:381–388.

44. Reuben RC, Roy PC, Sarkar SL, Alam Rubayet-Ul, Jahid IK (2019) Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. BMC Microbiol;19:253.

45. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

46. Salim A, Babu P, Mohan K, Moorthy M, Raj D, Thirumeni SK, Suresh S, Madhavan A, Nair B G, Chattopadhyay S, Pal S (2019) Draft Genome Sequence of an *Escherichia coli* Sequence Type 155
47. Savage DC (1992) Growth phase, cellular hydrophobicity, and adhesion in vitro of lactobacilli colonizing the keratinizing gastric epithelium in the mouse. Appl Environ Microbiol 58:1992–1995.

48. Sharma K, Sharma N, Sharma R (2017) An Evaluation of In-vitro Potential of Novel Lactobacillus paraplantarum KM0 (KX671558) Strain Isolated from Milk. Proc Indian Natn Sci Acad 83(3):689–699.

49. Sheila M, Fontaine O Peerce N (1992) Impact of rice based oral rehydration solution on stool output and duration of diarrhoea: meta-analysis of 13 clinical trials. BMJ Clinical Research 304:287–291.

50. Shokryazdan P, Sieo CC, Kalavathy R, Liang J B, Alitheen BN, Jahromi MF, Ho YW (2014) Probiotic Potential of Lactobacillus Strains with Antimicrobial Activity against Some Human Pathogenic Strains. BioMed Research International.

51. Sidira M, Kourkoutas Y, Kanellaki M, Charalampopoulos D (2015) In vitro study on the cell adhesion ability of immobilized lactobacilli on natural supports. Food Res Int;76 (3): 532-539.

52. Sorroche FG, Spesia MB, Zorreguieta Á, Giordano WA (2012) A positive correlation between bacterial autoaggregation and biofilm formation in native Sinorhizobium meliloti isolates from Argentina. Appl Environ Microbiol 78(12):4092–4101.

53. Spanhaak S, Havenaar R, Schaafsma G (1998) The effect of consumption of milk fermented by Lactobacillus casei strain Shirota on the intestinal microflora and immune parameters in humans. Eur J Clin Nutr 52(12):899-907.

54. Štyria I, Nemcová R, Chang YH, Ljungh A (2003) Binding of extracellular matrix molecules by probiotic bacteria. Lett Appl Microbiol 37:329–333.

55. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 101(30):11030–11035.

56. Toba T, Virkola R, Westerlund B, Bjorkman Y, Sillanpaa J, Vartio T, Kalkkinen N, Korhonen TK (1995) A collagen-binding S-layer protein in Lactobacillus crispatus. Appl Environ Microbiol 61(7):2467–2471.

57. Tuo Y, Yu H, Ai L, Wu Z, Guo B, Chen W (2013) Aggregation and adhesion properties of 22 Lactobacillus strains. J Dairy Sci 96(7):4252–4257.

58. Van Boeckel TP, Pires J, Silvester R, Zhao C, Song J, Criscuolo NG, Gilbert M, Bonhoeffer S, Laxminarayan R (2019) Global trends in antimicrobial resistance in animals in low and middle-income countries. Science 365(6459):eaaw1944.

59. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic Bacteria as Biological Control Agents in Aquaculture. Microbiol Mol Biol Rev 64(4):655–671.

60. Vijayakumar PP, Muriana PM (2015) A microplate growth inhibition assay for screening bacteriocins against Listeria monocytogenes to differentiate their mode of action. Biomolecules 5(2):1178-94.

61. Vlková E, Rada V, Šmehlová M, Killer J (2008) Autoaggregation and Coaggregation ability in Bifidobacteria and Clostridia. Folia Microbiol (Praha) 53(3):263-269.
62. Vuotto C, Longo F, Donelli G (2014) Probiotics to counteract biofilm-associated infections: promising and conflicting data. Int J Oral Sci 6(4):189-194.

63. Wong HB (1981) Rice water in treatment of infantile gastroenteritis. Lancet 2(8237):102-103.

64. Xu A, Mackay W, Scullen OJ, Sheen S, Ramos R, Sommers C (2019) Draft Genomic Sequence of *Escherichia coli* Sequence Type 131, Isolated from Retail Chicken Skin. Microbiol Resour Announc 8(7).

65. Xu Y, Tian Y, Cao Y, Li J, Guo H, Su Y, Tian Y, Wang C, Wang T, Zhang L (2019) Probiotic properties of *Lactobacillus paracasei* subsp. *Paracasei* L1 and its growth performance-promotion in chicken by improving the intestinal microflora. Front Physiol 10:937

66. Xue L, He J, Gao N, Lu X, Li M, Wu X, Liu Z, Jin Y, Liu J, Xu J, Geng Y (2017) Probiotics may delay the progression of nonalcoholic fatty liver disease by restoring the gut microbiota structure and improving intestinal endotoxemia. Sci Rep 7:45176

67. Yadav AK, Tyagi A, Kumar A, Saklani AC, Grover S, Batish V K (2015) Adhesion of indigenous *Lactobacillus plantarum* to gut extracellular matrix and its physicochemical characterization. Arch Microbiol 197:155–64.

68. Zhang H, Liu L, Hao Y Zhong S, Liu H, Han T, Xie Y(2013) Isolation and partial characterization of a bacteriocin produced by *Lactobacillus plantarum* BM-1 isolated from a traditional fermented chinese meat product. Microbiol Immunol 57(11):746-55.

69. Zhang J, Liu G, Shang N, Cheng W, Chen S, Li P (2009) Purification and Partial Amino Acid Sequence of Pentocin 31-1, an Anti-Listeria Bacteriocin Produced by *Lactobacillus pentosus*. J Food Prot. 72(12):2524-2529.

70. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7(1-2):203–214.

71. Zivkovic M, Miljkovic M, Ruas-Madiedo P, Strahinic I, Tolinacki M, Golic N, Kojic M (2015) Exopolysaccharide production and ropy phenotype are determined by two gene clusters in putative probiotic strain *Lactobacillus paraplantarum* BGCG11. Appl Environ Microbiol 81:1387–1396.

72. Zokaeifar H, Luis Balcázar J, Kamarudin MS, Sijam K, Arshad A, Saad CR (2012) Selection and identification of non-pathogenic bacteria isolated from fermented pickles with antagonistic properties against two shrimp pathogens. J Antibiot (Tokyo) 65(6):289-294.

73. Zubaidah E, Nurcholis M, Wulan SN, Kusumab A (2012) Comparative Study on Synbiotic Effect of Fermented Rice Bran by Probiotic Lactic Acid Bacteria *Lactobacillus casei* and Newly Isolated *Lactobacillus plantarum* B2 in Wistar Rats. APCBEE Procedia 2:170–177.

74. Zulfakar SS, White JD, Ross T, Tamplin ML (2012) Bacterial attachment to immobilized extracellular matrix proteins in vitro. Int J Food Microbiol 157:210–217.

**Figures**
Figure 1

Phylogenetic analysis of 16S rDNA partial genome sequences of the RS (a) and T1(b). The analysis was conducted with MEGA5 using neighbor-joining method. The optimal tree with the sum of branch length = 0.15357618 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 95 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1549 positions in the final dataset. Evolutionary analyses were conducted in MEGA X and top 10 hits are shown.
Figure 2

Probiotic properties of RS (L. fermentum MN410703) and T1 (L. fermentum MN410702) Viable counts of RS and T1 exposed to lower pH ranges after 3 h of exposure was measured ($P \leq 0.01$) (a-b). Viable counts of the strains after exposure to bile salts at 0.2 and 0.5% was determined ($P \leq 0.01$). (c).
Absorbance values of RS and T1 after exposure to simulated gastric juice (SGJ) at pH 2, 3 and 7 was determined (d-e). Values were expressed as mean ± SD of three individual experiments (Two-way RM ANOVA).

Figure 3

Elucidation of cell surface properties. Percentage of hydrophobicity exhibited by probiotic strains towards hexadecane and xylene was determined by MATH assay (a). Auto aggregation percentages of RS and T1 until 24 h was compared with B. clausii and E. coli ET (P ≤ 0.001) (b). Coaggregation percentages of RS and T1 strains with the pathogens, Salmonella enterica, E. coli MDR and K. pneumoniae after 24 h of incubation (P ≤ 0.0001) (c-d). Statistical analysis of data obtained was performed by conducting Two-way RM ANOVA.
Figure 4

Bacterial attachment to immobilized extracellular matrix proteins (ECM); Gelatin (a) (P ≤0.0001) and Heparin (b). Binding efficiency was compared with Bacillus clausii and E. coli ET. Statistical analysis of data obtained was performed by conducting Two-way RM ANOVA.
Figure 5

Fluorescent and Crystal violet stained images of RS and T1 in gelatin and heparin matrix. Crystal violet (CV) stained and acridine orange stained images of B. clausii (a-b), E. coli ET (c-d) T1 (e-f), and RS (g-h) binding to gelatin. CV stained and acridine orange stained images of B. clausii (i-j), E. coli ET (k-l), T1 (m-n) and RS (o-p) binding to heparin. Microbial adherence after 5 h of binding to the matrix was recorded and compared with B. clausii and E. coli ET.
Figure 6

Quantitative analysis of biofilm formation was performed in comparison with B. clausii (a). Biofilm formation ability of RS and T1 and crystal violet stained and acridine orange stained images of RS (b-c), and T1 (d-e). Qualitative determination of β-galactosidase activity of RS and T1 impregnating ONPG discs (f). Values were expressed as mean ± SD of three individual experiments (Two-way RM ANOVA).
Figure 7

Reduction of E. coli (coliform) counts in sewage. Biofilter impregnated with T1 and B. clausii was found to reduce coliforms by 1 log (90%) when immobilized in biofilter (P ≤ 0.01) in comparison with biofilter without microbial consortium. Values were expressed as mean ± SD of three individual experiments (Two-way RM ANOVA).
Figure 8

Percentage inhibition rates of pathogens treated with Bacteriocin like inhibitory substance (BLIS) from RS (P ≤ 0.0001). Controls employed was untreated bacterial culture. Values were expressed as mean ± SD of
three individual experiments (Two-way RM ANOVA).