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Evaluation of safety aspects, *in vitro* probiotic potential and anti-inflammatory activity of *Lactobacilli* isolated from Meghalayan traditional fermented rice beverage

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Two indigenous lactobacilli, *Limosilactobacillus fermentum* MTCC 25515 and *Lacticaseibacillus rhamnosus* M9 isolated from Indian traditional fermented rice beverage were studied for safety criteria, probiotic attributes by *in vitro* tests and anti-inflammatory activity in cell line. They were negative for biogenic amines production, gelatinase, lecithinase and hemolytic activity, and displayed moderately low antibiotic resistance. They survived at low pH and 0.5% bile. They remain viable under simulated gastric and intestinal juice. Cell surface hydrophobicity and cell autoaggregation ability of *Limosilactobacillus fermentum* MTCC 25515 were comparatively higher than *Lacticaseibacillus rhamnosus* M9. Cell coaggregation and antimicrobial activity were relatively high in *Lacticaseibacillus rhamnosus* M9 than *Limosilactobacillus fermentum* MTCC 25515. They had no bile salt hydrolase activity. *Limosilactobacillus fermentum* MTCC 25515 produced comparatively higher short chain fatty acids than *Lacticaseibacillus rhamnosus* M9. Additionally, the lipopolysaccharide-stimulated excessive production of proinflammatory cytokines and nitric oxide in RAW 267.4 cells was considerably reduced by *Limosilactobacillus fermentum* MTCC 25515.

Keywords: *Lacticaseibacillus rhamnosus* M9, *Limosilactobacillus fermentum* MTCC 25515, Probiotic, Proinflammatory cytokines, RAW 267.4 cells, Short chain fatty acid

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Elie Metchnikoff, a Nobel Prize winner, is largely credited with developing the original hypothesis of probiotics. In 1908, he postulated that the lactic bacteria in fermented milk of Balkan people might be responsible for their longer lifespan. Lactobacilli are generally considered beneficial microorganisms, with some strains even considered to promote good health *i.e.,* probiotic and their extensive historical use contributes to their acceptance as being generally recognized as safe (GRAS) for human consumption[1]. The administration of living microorganisms raises possible safety concerns that must be addressed before utilizing probiotics in food development and/or pharmaceutical preparation. Though having GRAS status, lactobacilli, may be the carrier of virulence factor. Pathogenicity and safety aspects of a particular strain are associated with antibiotics resistance, production of extracellular proteins/metabolites such as hemolysin, lecithinase, gelatinase, mucin degrading enzyme, biogenic amines and other virulence factors along with a surface proteins and aggregation substances[2,3]. The candidate probiotic lactobacilli should be free from pathogenic and virulent activity.

A strain has to satisfy specific physiological requirements in order to be recognized as a probiotic, such as viability in the gut, lower pH adaptability, bile resistance in the intestinal tract, antimicrobial activity, ability to reduce pathogen adhesion etc[4]. The human digestive tract secretes about 2.5 L of gastric juice[5] and 1 L of bile per day[6]. Thus, the ability of bacteria to remain alive in simulated oral, gastric and intestinal juices predicts the probiotic attribute under actual situation[7]. The potential for adhesion to intestinal mucus and epithelial cells is also an essential

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characteristic for an ideal probiotic. It can be achieved by 1) production of antimicrobial substances, thereby preventing pathogenic microbial proliferation and 2) adherence to mucus to prevent pathogen colonization[8].

Probiotic lactobacilli were found to produce short chain fatty acids (SCFA) during skim milk fermentation[9]. The presence of SCFA provide conducive environment for fermentation in the large intestine, restricts procarcinogens' absorption and reduces inflammation[10]. *Lactobacillus* strains have been reported to produce acetic acid, lactic acid and butyric acid in fermented skim milk medium[11]. Several *Lactobacillus* species are known to possess anti-inflammatory activity. Lactobacilli inhibited the expression of inflammatory genes before, after and during the initiation of inflammation[12]. According to previous studies, lactobacilli were able to reduce the level of proinflammatory cytokines like IL-6 and IL-1β, when added to sonicated pathogen exposed HT-29 cell line[13]. Reducing the concentration of proinflammatory cytokines could therefore have a beneficial effect by reducing inflammation in the host. The present study was conducted with an aim to check the safety aspect, probiotic potential and anti-inflammatory activity of two potent lactobacilli strains isolated from Meghalayan traditional fermented rice beverage. The Garo tribes are major inhabitants of North Eastern part of India and many traditional fermented foods of this tribe were found rich with healthy bacteria[14,15]. The lactobacilli isolated from such foods with probiotic potential could be utilised in the preparation of biofunctional foods.

Materials and Methods

Bacterial strains purity and maintenance

Limosilactobacillus fermentum MTCC 25515 and *Lacticaseibacillus rhamnosus* M9 isolated from Indian traditional fermented rice beverage were analysed for safety aspects and probiotic potential. Probiotic strain, *Lactobacillus helveticus* MTCC 5463 was used as positive control. *Limosilactobacillus fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 were propagated in MRS broth and indicator organisms (*Bacillus cereus* MTCC 1272, *Staphylococcus aureus* MTCC 737, *Enterococcus fecalis* ATCC 29212, *Escherichia coli* MTCC 1678, *Salmonella enterica* ser. Typhimurium ATCC 14028 and *Salmonella enterica* ser. Paratyphi MTCC 735) in nutrient broth and incubated at 37°C for 15 h. Purity of bacterial strains was ascertained prior to use by microscopic (1000X) examination through Gram staining and catalase test.

Safety test for Lactobacilli

The disc diffusion assay was used to assess antibiotic sensitivity of lactobacilli by following the reported method[16]. Lactobacilli were evaluated for production of biogenic amines by following the reported method[17]. Gelatinase activity of lactobacilli was tested by following the method of Zhang *et al.*[18]. Lecithinase activity of lactobacilli was checked by following the method of Mohkam *et al.*[19]. Hemolytic activity in lactobacilli was checked by following the method of Zhang *et al.*[18].

In vitro probiotic tests

Kathiriya *et al.*[20] method was followed for pH and bile tolerance. After being propagated in MRS medium, the lactobacilli were left to grow for 15 hours at 37°C to activate them. Then centrifuged at 10,000 rpm/10 min (Eppendorf Centrifuge, US) and pellets were suspended in phosphate buffer saline (PBS) and again centrifuge to wash the cells then, re-suspended into PBS. For pH tolerance, cell suspension was added at the rate of 2% to 10 mL MRS broth adjusted to varying pH (1.5, 3 and 6.5). Samples were drawn at 0 h, 1.5 h and 3 h. For bile tolerance, cell suspension was added at the rate of 2% to 10 mL MRS broth with 0.5% (w/v) ox-bile and control (no bile added) and incubated at 37°C. Samples were drawn at 0 h, 2 h and 4 h. The effect of simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) on lactobacilli was studied according to Guantario *et al.*[21].

Cell surface hydrophobicity (CSH) and Cell auto-aggregation ability were determined as per the reported procedures[20]. Cell suspension was adjusted to 0.25±0.05 (A_0) optical density (OD) at 600 nm using PBS. For CSH, equal proportion of cell suspension and Xylene were taken and mixed vigorously for 2 min on vortex mixture and placed at 37 °C for 1 h. The OD of aqueous phase was determined (A_1).

$$\% \text{ Hydrophobicity} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

For cell autoaggregation ability, 5 mL of cell suspension was taken into clean and dry test tube, mixed by vigorous vortexing for 1 min. After 5 h without disturbing bacterial suspension, 3 mL of the upper phase was taken for recording OD_{600} (A_5).

$$\% \text{ Autoaggregation} = \left(\frac{A_0 - A_5}{A_0}\right) \times 100$$

The procedure adopted by Kathiriya *et al.*[20] was considered for cell coaggregation ability. *Bacillus cereus* MTCC 1272, *Staphylococcus aureus* MTCC 737, *Enterococcus fecalis* ATCC 29212, *Escherichia coli* MTCC 1678, *Salmonella enterica* ser. Typhimurium ATCC 14028 and *Salmonella enterica* ser. *paratyphi* MTCC 735 were used to check the coaggregation ability of lactobacilli. The method for preparing the bacterial suspension for lactobacilli and indicator organism was same as that of pH tolerance. The OD_{600} of each bacterial suspension of lactobacilli (A_l) and indicator organisms (A_p) was adjusted to 0.25±0.05. Equal volumes of lactobacilli and indicator organism suspension mixed for 1 min on vortex mixture. After 5 h of incubation, upper layer was taken for OD_{600} (A_5) measurement.

$$\% \text{ Coaggregation} = \left(\frac{\left(\frac{A_l + A_p}{2}\right) - A_5}{\frac{A_l + A_p}{2}}\right) \times 100$$

Where,

A_l = OD_{600} of individual lactobacilli bacterial suspension at 0 h

A_p = OD_{600} of individual indicator organism suspension at 0 h

A_5 = OD_{600} of mixture of one lactobacillus and one indicator organism suspension after 5 h

The antimicrobial activity of the lactobacilli was tested by the agar well diffusion method[20] against *Bacillus cereus* MTCC 1272, *Staphylococcus aureus* MTCC 737, *Enterococcus fecalis* ATCC 29212, *Escherichia coli* MTCC 1678, *Salmonella* Typhimurium ATCC 14028 and *Salmonella enterica* ser. Paratyphi MTCC 735.

For bile salt hydrolase (BSH) activity, lactobacilli were streaked on MRS agar with 0.3% (w/v) bile salts. The petri plates incubated anaerobically at 37°C for 4 days. The cholic acid precipitated around the colony indicates presence of bile salt hydrolase[22].

Short chain fatty acids production

Production of SCFA in milk medium was also determined[23]. The active lactobacilli were added at the rate of 2% into the sterile 12% reconstituted skim milk and then incubated at 37ºC for 24 h. Two milliliters of sample and 7 mL 10 mM NaOH containing 0.1 mM crotonic acid was taken into 15 mL centrifuge tube and placed in shaker incubator at 30°C for 6 h. After incubation, 1 mL chloroform was added to remove fat soluble substance and centrifuged at 10,000 rpm for 15 min. The supernatant recovered was filtered using 0.22 µm membrane. The filtered sample injected into HPLC for the detection of SCFA. For HPLC analysis, 0.1% phosphoric acid isocratic mixture was taken as mobile phase. The column was cleaned two times using 0.1% phosphoric acid to remove contaminants. Following a column wash, the organic acid was eluted with 0.1% phosphoric acid at a flow rate of 0.2 mL/min while the oven was maintained at 30°C. The absorbance was measured at 210 nm.

Cell line study

Cell culture collection and viability

National Cell Science Center, Pune, (Maharashtra, India) supplied RAW 264.7 cells. From Lonza, Bioscience (Switzerland), the Dulbecco's modified Eagle's medium (DMEM) was purchased. Fetal Bovine Serum (FBS) obtained from MP Biomedicals. Cusabio Biotech (China) provided the Lipopolysaccharide (LPS). Proinflammatory cytokines were measured by ELISA assay (Elabscience, USA). RAW 264.7 cells were subcultured after growing on DMEM containing 10% FBS and 1% Penicillin/streptomycin (P/S) at 2 days intervals. MTT assay was performed according to Khare *et al.*[24]. About 5×10^3 cells/well seeded into 96-well plates and incubated at 37°C for 16 h. *Limosilactobacillus fermentum* MTCC 25515 at 8, 6, 4, 2, 1, 0.5 mg/mL and MTT was diluted to 0.5 mg/mL. The plate was then incubated for 4 h at 37°C and 5% CO_2 in the dark at 37°C and 5% CO_2. For the dissolution of formazan crystals, medium was removed carefully followed by addition of 0.1 mL of DMSO. Further, absorbance was determined at 570 nm using a microplate reader (M200 PRO, Tecan Life Science).

Nitric Oxide (NO) and TNF-α, IL-6, & IL-1β Cytokines Production

RAW 264. 7 cells (1×10^5 cells) were seeded in a 48-well plate and incubated for 24 h. The confluent macrophages were treated with 1 µg/mL LPS with or without *L. fermentum* MTCC 25515. Additional 16 h were used for incubating the cells in a humidified CO_2 incubator. The supernatant was then collected, and nitrite concentration was determined using Griess reagent, followed by OD at 540 nm. The levels of TNF-α, IL-6, and IL-1β of cell-free supernatant were measured using ELISA assay (Elabscience, USA).

Statistical analysis

All results are mean of three independent experiments and expressed as mean ± standard deviation (mean±SD). One- or two-way analysis of variance (ANOVA) was applied and comparison was made at 5% significance level using completely randomized design. One-way ANOVA was applied in the cell line investigation to assess different treatments and then Tukey's post hoc test was performed. The data were analyzed using Graph Pad Prism 8.0 Software Inc. (La Jolla, CA, USA).

Results and Discussion

Safety tests for Lactobacilli

Presence of mobile antibiotic resistance gene and acquired antibiotic resistance in probiotic lactobacilli is considered dangerous as it could transfer acquired antibiotic resistant genes to harmful bacteria but presence of intrinsic antibiotic resistance genes in the candidate probiotic strains is beneficial as they can survive the adverse conditions and can be used along with the antibiotic treatment in the host. Zone of inhibition (mm) produced by *L. fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 against various antibiotic discs using disc diffusion assay is shown in Table 1. All lactobacilli strains had varying level of resistance to a particular antibiotic. All lactobacilli strains were found sensitive to the antibiotics like ampicillin, erythromycin, rifampicin gentamicin, streptomycin and tetracycline. In addition to these antibiotics, *L. fermentum* MTCC 25515 was also sensitive to methicillin and vancomycin while, *L. rhamnosus* M9 was sensitive to ciprofloxacin. The three strains were resistant to oxacillin, kanamycin and norfloxacin that interrupt either protein biosynthesis or cell wall biosynthesis or DNA biosynthesis in the bacteria regardless of their source of origin. Mayrhofer *et al.*[25] reported that lactobacilli had intrinsic resistance to various aminoglycosides (kanamycin, streptomycin) and vancomycin[26]. So, the kanamycin resistance by all the three strains and vancomycin resistance by *L. rhamnosus* M9 and *L. helveticus* MTCC 5463, in the study could be due to this phenomenon.

Biogenic amines (BA) are organic bases of low molecular weight, polar or semi-polar compounds, resulting from the decarboxylation of amino acids and ingestion of food containing high BA can cause headache, heart palpitations, vomiting, diarrhoea and hypertensive crises. None of the lactobacilli were able to produce BAs from ornithine and arginine amino acid substrate, however, positive control (*E. coli* and *Salmonella typhi*) showed positive result for the BA production (Supplementary Fig. S1). Our results were in agreements with published study[27], *L. rhamnosus* R0011, *L. rhamnosus* Lr-32 and *L. paracasei* Lpc-37 strains did not decarboxylate L-Arginine, L-Histidine, L-Lysine, L-Tyrosine, and L-Tryptophan. In another study, *L. pentosus* CHIG, *L. pentosus* NAG1 and *L. fermentum* PRS1 from plant source did not produce biogenic amines[28].

Table 1 — Antibiotic susceptibility of lactobacilli (as per the guidelines of Clinical and Laboratory Standards Institute)

Antibiotics (Concentration, µg/disc)	Zone of inhibition (mm)			Treatment Mean (Antibiotics)
	Limosilactobacillus fermentum MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463	
Ampicillin (10)	14.50±0.71	11.00±1.41	10.50±0.71	12.00
Oxacillin (1)	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Ciprofloxacin (5)	0.00±0.00	11.00±0.00	0.00±0.00	3.67
Erythromycin (15)	24.00±1.41	25.00±1.41	23.00±1.41	24.00
Rifampicin (5)	22.00±1.41	26.00±1.41	26.50±0.71	24.83
Kanamycin (30)	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Norfloxacin (10)	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Methicillin (5)	14.00±0.00	0.00±0.00	0.00±0.00	4.67
Vancomycin (30)	21.50±0.71	0.00±0.00	0.00±0.00	7.17
Gentamycin (10)	13.00±1.41	12.50±0.71	13.00±1.41	12.83
Streptomycin (10)	19.50±0.71	10.50±0.71	9.50±0.71	13.17
Tetracycline (30)	28.50±0.71	27.50±0.71	20.50±0.71	25.50
Period Mean (Lactobacilli)	13.08	10.29	8.58	

CD (0.05) T=0.45, P=0.91, T×P=1.57; CV (%) =7.25

Diameter of the inhibition zone including disc diameter, antibiotic disc diameter=6 mm, Values are presented as mean±SD (n= 3) and evaluated by two-way ANOVA using completely randomised design. CD (0.05) =Critical difference at 5% level of significance; CV (%) =Percent coefficient of variance

The gelatinase would interfere with the normal functioning of the mucoid lining of the gastro intestinal tract (GIT) and these injuries would be the pathways for infection. Hence, the potential probiotic bacteria must be negative for gelatinase activity. Both lactobacilli strains *i.e., L. fermentum* MTCC 25515 and *L. rhamnosus* M9, had no gelatinase activity (Supplementary Fig. S2), while positive control, *Staphylococcus aureus* showed clear zone around the colony after flooding the Petri dish with saturated ammonium sulphate solution. The results were in accordance with previously published studies where gelatinase activity was not observed in *L. rhamnosus* AD3 and *L. rhamnosus* GG[29], *L. rhamnosus* CRL 1332[30], *L. plantarum* A41, *L. fermentum* SRK414, *L. brevis* K35, *L. gasseri* CKDB 027, *L. gasseri* CKDB 020, *L. acidophilus* CKDB 009, *L. acidophilus* CKDB 013, *L. reuteri* CKDB 030[31].

Lecithinase enzymes secreted by microorganisms, act by facilitating the invasion of tissues. So, lecithinase activity should be absent in the probiotic bacteria. *Limosilactobacillus fermentum* MTCC 25515 and *L. rhamnosus* M9 were found negative for lecithinase activity while, *Staphylococcus aureus* showed lecithinase precipitation as well as clear halo formation around the colony (Supplementary Fig. S3). The earlier investigations discovered similar outcomes. Lecithinase activity was absent in *L. fermentum* 2pr., *L. fermentum* 11 d.st., *L. fermentum* 11 zv., *L. rhamnosus* 7 d.st., *L. rhamnosus* 24 d.st., *L rhamnosus* 38 k and *L. rhamnosus* 32 k[32], and *L. rhamnosus* D[33].

Hemolytic activity is considered as a tissue-damaging virulence factor in our body. That's why, probiotic bacteria must be negative for the hemolytic activity. Both the lactobacilli did not show hemolytic activity on MRS agar containing 5% sheep blood where as positive control *Staphylococcus aureus* showed clear zone around the colonies on nutrient agar containing 5% sheep blood indicating positive for β-hemolytic activity (Supplementary Fig. S4). Our results of hemolytic activity were in agreement with the following reported studies. Hemolytic activity was not found for *L. rhamnosus* AD3, *L. rhamnosus* GG[29], *L. rhamnosus* CRL 1332[30], *L. pentosus* CHIG, *L. pentosus* NAG1 and *L. fermentum*[28].

***In vitro* probiotic tests**

Low pH in stomach (pH 1.5-3.5) is one of the constraints for bacteria to survive and multiply further as acidic pH in stomach kills viable microflora consumed by the hosts along with the food[7]. Hence, in order to prove a bacterium as potential probiotic, it should tolerate low pH and grow in optimum numbers to provide probiotic effects. Viable lactobacilli count of each *L. fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 strains were determined for 0, 1.5 h, and 3 h of incubation under pH 1.5 and pH 3. The lactobacilli found more resistant at pH 3 than pH 1.5. The *L. fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 could not tolerate pH 1.5, while survived at pH 3.5 for 3 h (Table 2). It was observed that *L. rhamnosus* M9 was more resistant to low pH, followed by *L. helveticus* MTCC 5463 and *L. fermentum* MTCC 25515. The ability of lactobacilli to survive at such a low pH predicts their ability to remain alive in GIT. Following studies reported the similar results. When *L. rhamnosus* NK2, *L. casei* NK9, *L. rhamnosus* NK10, *L. pentosus* M20 and *L. plantarum* M22 were exposed to pH 3, the viable

Table 2 — Viability (log CFU/mL) of lactobacilli in MRS broth at different pH for various time intervals

Time	*Limosilactobacillus fermentum* MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463
pH			
0 h			
6.5	7.39±0.16^{a}	7.65±0.22^{a}	7.55±0.22^{a}
3.0	7.45±0.15^{a}	7.67±0.14^{a}	7.47±0.41^{a}
1.5	7.52±0.25^{a}	7.66±0.27^{a}	7.53±0.36^{a}
1.5 h			
6.5	7.40±0.27^{a}	7.72±0.24^{a}	7.62±0.27^{a}
3.0	6.38±0.23^{b}	6.61±0.27^{b}	6.48±0.32^{b}
1.5	3.39±0.20^{d}	4.61±0.29^{d}	4.53±0.26^{d}
3h			
6.5	7.59±0.22^{a}	7.81±0.15^{a}	7.82±0.36^{a}
3.0	5.15±0.21^{c}	5.42±0.20^{c}	5.25±0.35^{c}
1.5	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}

Values are mean±SD (n=3) and evaluated by two-way ANOVA using completely randomised design. Values with different superscripts in each column differ significantly ($p<0.05$)

count as log CFU/mL, was 7.40, 7.27, 7.34, 7.25 and 7.22, respectively at 0 h which reduced to 6.94, 6.36, 4.96, 5.37 and 6.77, respectively, after 3 h[34]. In another study, the viable count as log CFU/mL of *L. rhamnosus* K4E and *L. fermentum* K16 reduced from 8.83 and 8.29, respectively at 0 h to 4.65 and 5.12, respectively at 4 h, when exposed to pH 2[35].

After the acidic barrier of the stomach, bacteria are exposed to bile fluid. Therefore, resistance to bile salts is considered as an important parameter for selecting probiotic strains. Bile act as emulsifier, dissolves the cell membrane of living cell thereby, disrupting the homeostasis. During first hour of digestion about 0.5-2% bile is present in the intestine[36]. It was observed that *L. fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 were able to survive at 0.5% oxgall concentration (Table 3). Cell concentration of all the lactobacilli in control (does not contain bile salt) and broth containing 0.5% ox gall were at par ($p>0.05$) after 4 h of exposure. In a similar study, *L. fermentum* DUR 18 isolated from human milk, survived at 0.3% bile salt for 3 h[37]. *Limosilactobacillus fermentum* MA-8, cell count as log CFU/mL increased from 8.03 to 8.54 when exposed to 0.3% bile for 4 h[38]. *Lacticaseibacillus rhamnosus* NS6 cells count as log CFU/mL increased significantly ($p<0.05$) when exposed to 0.5% bile in MRS broth, from 5.24 at 0 h to 5.67 at 4 h[34].

Probiotics have a pivotal role on microbial balance and protection of the digestive system. Probiotic bacteria should be capable of withstanding extreme stomach conditions and adhere to epithelial cells of GIT. The effect of SGJ on viability of *L. fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 is given in Table 4. All lactobacilli were found to survive in SGJ during 3 h of exposure. A significant ($p<0.05$) reduction in lactobacilli count of all the three cultures was observed in SGJ compared to control, after 3 h. Das *et al.*[35] also found that the count as log CFU/mL of *L. rhamnosus* K4E and *L. fermentum*, reduced significantly ($P<0.05$) from 8.23 and 8.15, respectively at 0 h to 5.30 and 5.10, respectively at 4 h, when exposed to SGJ (NaCl; KCl; $NaHCO_3$ and pepsin, pH 3). *Lacticaseibacillus rhamnosus* GG, *L. rhamnosus* FS2 and *L. paracasei* PM8 count as log CFU/mL under SGJ (0.2% pepsin in 0.5% sterile saline, pH 2.5) was found to reduce from 9.21, 9.41 and 9.54, respectively at 0 h to 8.62, 8.62 and 8.84, respectively at 3 h[39].

Along with the bile salts, pancreatic enzymes are also found in the intestinal fluid which helps in digestion of food. The two lactobacilli strains *L. fermentum* MTCC 25515 and *L. rhamnosus* M9, along with control (*L. helveticus* MTCC 5463) were tested for SIJ tolerance ability. All the three lactobacilli were found to survive in SIJ during 4 h of

Table 3 — Viability (log CFU/mL) of lactobacilli in MRS broth containing bile salt at different time intervals

Time / Bile	*Limosilactobacillus fermentum* MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463
0 h			
0.0%	7.38±0.23[a]	7.47±0.29[a]	7.50±0.40[a]
0.5%	7.31±0.28[a]	7.44±0.31[a]	7.34±0.23[a]
2 h			
0.0%	7.51±0.23[a]	7.58±0.25[a]	7.65±0.30[a]
0.5%	7.40±0.20[a]	7.52±0.27[a]	7.48±0.31[a]
4 h			
0.0%	7.99±0.26[a]	8.05±0.31[a]	8.08±0.31[a]
0.5%	7.77±0.27[a]	7.91±0.26[a]	7.77±0.23[a]

Values are mean±SD (n=3) and evaluated by two-way ANOVA using completely randomised design. Values with different superscripts in each column differ significantly ($p<0.05$)

Table 4 — Viability (log CFU/mL) of lactobacilli in simulated gastric juice (SGJ) at different time intervals

Time	*Limosilactobacillus fermentum* MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463
0 h			
0.85% saline	7.64±0.33[a]	7.30±0.26[a]	7.42±0.24[a]
SGJ	7.67±0.29[a]	7.59±0.22[a]	7.57±0.20[a]
3 h			
0.85% saline	7.99±0.25[a]	7.57±0.35[a]	7.68±0.35[a]
SGJ	4.28±0.22[b]	4.53±0.17[b]	4.39±0.34[b]

Values are mean±SD (n=3) and evaluated by two-way ANOVA using completely randomised design. Values with different superscripts in each column differ significantly ($p<0.05$)

exposure period; lactobacilli count were found to increase slightly with non-significant difference between them, after 4 h of exposure and were also found to be at par with control (0.85% saline) (Table 5). This means that there was no significant effect of SIJ on these three lactobacilli strains. Similar results were reported in another study where *Lacticaseibacillus rhamnosus* FS2 and *L. paracasei* PM8 count as log CFU/mL under SIJ (250 mg/L pancreatin from porcine pancreas and 0.45% porcine bile extract in 0.5% saline pH 7.5) was found to increase from 9.43 and 9.63, respectively at 0 h to 9.50 and 9.78, respectively at 4 h[39].

The adherence of probiotics to the epithelial cells lining of intestine is determined by the hydrophobicity of the bacterial cell surface *in vitro*, higher the hydrophobicity means greater adhesion. Tyfa *et al.*[40] classified bacterial strains into three categories: very hydrophobic (>50%), moderately hydrophobic (20% to 50%), and hydrophilic (<20%) based on the degree of adherence to hydrocarbons. Our lactobacilli fall under the category of moderately hydrophobic (Table 6). The *L. helveticus* MTCC 5463 showed significantly ($p<0.05$) higher cell surface hydrophobicity, followed by *L. fermentum* MTCC 25515 and *L. rhamnosus* M9. Our results were in agreement with the reported studies. Cell surface hydrophobicity of *L. rhamnosus* K4E, *L. fermentum* K3A and *L. fermentum* K5, was about 70%, 45% and 34%, respectively[35]. The cell surface hydrophobicity of *L. rhamnosus* NS6 to hydrocarbons like n-hexadecane and xylene was 36.90% and 43.64%[20].

Bacterial autoaggregation means aggregation of same type of cells which helps their persistence in the intestine. Aggregation also prevents the adhesion of pathogen and consequently protect the GIT of the host. The cell autoaggregation ability of *L. fermentum* MTCC 25515, *L. rhamnosus* M9 and *L. helveticus* MTCC 5463 were 22.32%, 19.04% and 25.47%, respectively after 5 h of incubation at 37°C. The *L. helveticus* MTCC 5463 showed significantly ($P<0.05$) higher cell autoaggregation ability followed by *L. fermentum* MTCC 25515 and *L. rhamnosus* M9 (Table 6). Three strains of *L. rhamnosus* isolated from cheese, showed cell autoaggregation in the range of 10 to 20%[39].

Many healthy bacteria have ability to coaggregate with harmful bacteria, thereby disfavouring attachment of harmful bacteria with intestinal receptor sites. Irrespective of indicator organism, the coaggregation ability was highest in *L. rhamnosus* M9, followed by *L. fermentum* MTCC 25515 and *L. helveticus* MTCC 5463 at 5 h of incubation (Table 7). It was reported that the coaggregation ability of *L. rhamnosus* K4E and *L. fermentum* K7 with *Salmonella typhi, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes* and *Escherichia coli* was 14.83%, 21.33%, 21.50%, 24.50% and 26.33%, respectively and 15.83%, 20.55%, 22.50%, 16.66% and 24.51%, respectively[35]. Kathiriya *et al.*[20] reported that the coaggregation ability of *L rhamnosus* NS6 with indicator strains, *Salmonella typhi, Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* was 33.95%, 33.72%, 33.24% and 45.30%, respectively. Four strains of *L. rhamnosus*, showed cell coaggregation with *E. coli* 555, in the range of 10 to 20%[39].

Probiotic strains must produce antimicrobial substances that exhibit antagonistic action toward

Table 5 — Viability (log CFU/mL) of lactobacilli in simulated intestinal juice (SIJ) at different time intervals

Time	*Limosilactobacillus fermentum* MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463
0 h			
0.85% saline	7.41±0.25[a]	7.68±0.22[a]	7.59±0.33[a]
SIJ	7.42±0.28[a]	7.61±0.23[a]	7.62±0.29[a]
4 h			
0.85% saline	7.88±0.21[a]	8.16±0.29[a]	8.08±0.27[a]
SIJ	7.52±0.28[a]	7.73±0.29[a]	7.76±0.25[a]

Values are mean±SD (n=3) and evaluated by two-way ANOVA using completely randomised design. Values with different superscripts in each column differ significantly ($p<0.05$)

Table 6 — Percent hydrophobicity and cell autoaggregation of lactobacilli

Lactobacilli	Hydrophobicity (%)	Cell autoaggregation (%)
Limosilactobacillus fermentum MTCC 25515	40.78±1.28[b]	22.32±1.37[b]
Lacticaseibacillus rhamnosus M9	34.22±1.58[c]	19.04±1.41[c]
Lactobacillus helveticus MTCC 5463	45.63±2.05[a]	25.47±1.66[a]

Values are mean±SD (n=3) and evaluated by one-way ANOVA using completely randomised design. Values with different superscripts in each column differ significantly ($p<0.05$)

pathogen. *Limosilactobacillus fermentum* MTCC 25515 produced bigger inhibition zone against *Bacillus cereus* MTCC 1272, followed by *Enterococcus fecalis* ATCC 29212, *Staphylococcus aureus* ATCC 737, *Salmonella* typhimurium ATCC 14028, *Escherichia coli* MTCC 1687 and *Salmonella enterica* MTCC 735. Whereas, *L. rhamnosus* M9 produced maximum inhibition zone against *Bacillus cereus* MTCC 1272, followed by *Escherichia coli* MTCC 1687, *Salmonella enterica* MTCC 735, *Salmonella* typhimurium ATCC 14028, *Enterococcus fecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 737. Irrespective of indicator organisms, *L. rhamnosus* M9 exhibited highest ($p<0.05$) antimicrobial activity than *L. fermentum* MTCC 25515 and *L. helveticus* MTCC 5463 (Table 8). Similar study conducted by Das *et al.*[35] reported the zone of inhibition (mm) produced by *L. rhamnosus* K4E and *L. fermentum* K7 against *Bacillus cereus, Enterococcus fecalis, Shigella dysenteriae, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes* and *Salmonella typhi* was about 19 mm, 21 mm, 29 mm, 24 mm, 26 mm, 14 mm and 21 mm, respectively and 16 mm, 16 mm, 19 mm, 26 mm, 24 mm, 19 mm and 21 mm, respectively. While Kathiriya *et al.*[20] found that, *L. rhamnosus* NS6 had maximum antimicrobial activity followed by *S. thermophilus* MD2 and *S. thermophilus* MD8 against *E. coli, S. aureus, B. cereus* and *S. typhi*. Antimicrobial activity of *L. fermentum* MTCC 25515 and *L. rhamnosus* M9 against various indicator organisms showed the capability of tested lactobacilli to inhibit the growth of indicator organisms.

The lactobacilli *L. fermentum* MTCC 25515 and *L. rhamnosus* M9 were able to grow in MRS agar containing 0.3% taurodeoxycholate (TDC), glycocholate (GC), glycochenodeoxycholate (GCDC), when kept at 37°C for 4 days anaerobically. This indicated ability of these strains to tolerate above mentioned bile salts at given concentration. However, none of them were BSH positive as there were no opaque halo around colonies due to bile acid precipitation and could not form opaque granular white colonies with silvery shine (Supplementary Fig. S5). Similar results were obtained in our previous study where BSH activity was not observed in *Streptococcus thermophilus* MD2, *Streptococcus thermophilus* MD8

Table 7 — Cell coaggregation ability of lactobacilli with various indicator organisms

Name of indicator organisms	% Cell co-aggregation			Treatment Mean (Indicator organism)
	Limosilactobacillus fermentum MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463	
Bacillus cereus MTCC 1272	22.40±1.70	26.26±1.51	15.08±1.82	21.24
Staphylococcus aureus ATCC 737	22.92±1.77	27.81±1.04	16.14±0.87	22.29
Enterococcus fecalis ATCC 29212	15.54±1.78	18.57±0.63	12.89±0.91	15.66
Salmonella Typhimurium ATCC 14028	11.16±1.20	15.49±1.17	8.82±1.62	11.82
Escherichia coli MTCC 1687	15.80±1.43	19.75±0.99	12.41±1.37	15.99
Salmonella enterica MTCC 735	10.44±0.49	15.10±0.44	7.26±0.35	10.93
Period Mean (Lactobacilli)	16.38	20.50	12.10	

CD (0.05) T=0.77, P=0.55, T×P=1.34; CV%=7.74

Values are presented as mean±SD (n= 3) and evaluated by two-way ANOVA using completely randomised design. CD (0.05) =Critical difference at 5% level of significance; CV (%) =Percent coefficient of variance

Table 8 — Antimicrobial activity of lactobacilli against indicator organisms

Name of indicator organisms	Zone of inhibition (mm)			Treatment Mean (Indicator organisms)
	Limosilactobacillus fermentum MTCC 25515	*Lacticaseibacillus rhamnosus* M9	*Lactobacillus helveticus* MTCC 5463	
Bacillus cereus MTCC 1272	13.33±0.58	15.33±0.58	10.33±0.58	13.00
Staphylococcus aureus ATCC 737	12.00±1.00	11.67±0.58	14.33±0.58	12.67
Enterococcus fecalis ATCC 29212	13.17±0.29	13.00±1.00	11.00±1.00	12.39
Salmonella Typhimurium ATCC 14028	12.00±1.00	13.17±0.29	11.00±0.00	12.06
Escherichia coli MTCC 1687	11.67±0.58	15.00±0.00	13.67±0.58	13.44
Salmonella enterica MTCC 735	11.00±0.00	14.00±1.00	14.67±0.58	13.22
Period Mean (Lactobacilli)	12.19	13.69	12.50	

CD (0.05) T=0.63, P=0.45, T×P=1.09; CV%=5.15

Diameter of the inhibition zone including well diameter, well diameter=8 mm, values are presented as mean±SD (n= 3) and evaluated by two-way ANOVA using completely randomised design. CD (0.05) =Critical difference at 5% level of significance; CV (%) =Percent coefficient of variance

and *L. rhamnosus* NS6[20]. Four lactobacilli were BSH negative with taurocholate while one lactobacillus *i.e., L. plantarum* M22 was positive for BSH reaction[34]. In contrast to our result, Das *et al.*[35] reported that the five isolates *i.e., L. fermentum* K3A, *L. fermentum* K7*, L. fermentum* K16*, L. fermentum* K5 and *L. rhamnosus* K4E showed a positive BSH activity. The negative BSH activity in our lactobacilli, could be because of their source of isolation (fermented foods) as the BSH action was detected mostly in bacteria from mammal faeces or intestines that were high in bile acids.

Short chain fatty acids production

The significance of SCFA in the GIT and related health benefits have recently gained enormous attention due to rising health awareness[41]. *Limosilactobacillus fermentum* MTCC 25515 produced comparatively higher SCFA *i.e.,* 7.33 µg/mL acetic acid, 4.90 µg/mL propionic acid and 1.05 µg/mL butyric acid respectively than *L. rhamnosus* M9, which produced 2.75 µg/mL acetic acid, 1.54 µg/mL propionic acid and 0.80 µg/mL butyric acid, respectively. The control strain *L. helveticus* MTCC 5463 produced 2.22 µg/mL acetic acid, 1.25 µg/mL propionic acid and 0.50 µg/mL butyric acid, respectively (Fig. 1). Almost similar SCFA were obtained in the study where *Lacticaseibacillus rhamnosus* RNS4 produced 15.41 µg/mL acetic acid, 5.18 µg/mL lactic acid and 0.16 µg/mL butyric acid, while *L. fermentum* KGL2 produced 12.9 µg/mL acetic acid, 4.39 µg/mL lactic acid and 0.23 µg/mL butyric acid, respectively[11]. *L. rhamnosus* GG produced propionic acid (89 µM) in MRS medium as reported by LeBlanc *et al.*[41].

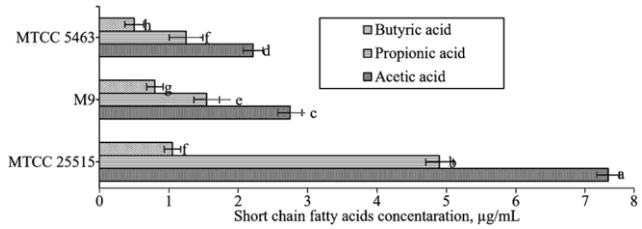

Fig. 1 — Short chain fatty acids (µg/mL) produced by lactobacilli in skim milk after 24 h. Data are presented as mean±SD (n= 3) and evaluated using two-way ANOVA by completely randomised design. Means with different lowercase letters (a, b, c, d, e, f, g, h) indicating significant difference ($p<0.05$)

Cell line study

Effect of L. fermentum MTCC 25515 on RAW 264.7 cells viability

As *L. fermentum* MTCC 25515 exhibited overall superior probiotic potential compared to *L. rhamnosus* M9, it was tested against RAW 264.7 cell line. The varying amount of *L. fermentum* MTCC 25515 *i.e.,* 8, 6, 4, 2, 1 and 0.5 mg/mL, was taken to check the cytotoxic effect of *L. fermentum* MTCC 25515 on the RAW 264.7 cells. At 2, 1 and 0.5 mg/mL *L. fermentum* MTCC 25515 treatment, there was no cytotoxicity observed on cell line (Fig. 2). Therefore,

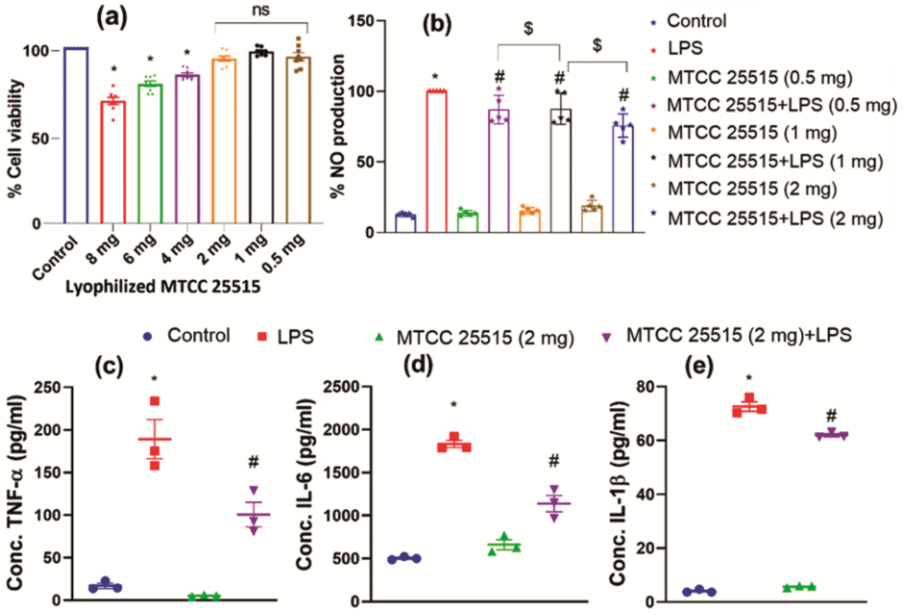

Fig. 2 — Effect of the *Limosilactobacillus fermentum* MTCC 25515 on (a) Cell viability (MTT assay) of RAW 264.7 cells (b) Nitric oxide (NO) production in *L. fermentum* MTCC 25515 (c) TNF-α (d) IL-6 (e) IL-1β measured in the supernatants of LPS-stimulated RAW 264.7 cells. Data are presented as mean ± SEM; n= 3 and evaluated by one-way ANOVA followed by Tukey's post hoc test. * relative to the control, # relative to the LPS, LPS- lipopolysaccharide. p value for # is 0.01, 0.0003 and 0.0002 in TNF-α, IL-6 and IL-1β respectively

doses of 2, 1 and 0.5 mg/mL were selected for the subsequent NO assay.

Effect of LPS-induced NO production in RAW 264.7 cells treated with L. fermentum MTCC 25515

The LPS treatment of macrophages led to significant increase of NO. It was curtailed using treatment of *L. fermentum* MTCC 25515 by 2, 1 as well as 0.5 mg/mL (Fig. 2). However, the 2 mg/mL dose was more effective in reducing NO than the 0.5 and 1 mg/mL doses, suggesting that this concentration is suitable for further experiments. The ability of *L. fermentum* MTCC 25515 to lower down LPS-induced NO production showed its potential anti-inflammatory activity. The dosage of 2 mg/mL *L. fermentum* MTCC 25515 was taken to study the effect on pro-inflammatory cytokine.

Cytokine analysis in RAW 264.7 cells

The TNF-α, IL-6, & IL- 1β levels were found to rise after LPS-stimulated macrophages and *L. fermentum* MTCC 25515 arrested such rise in levels of pro-inflammatory cytokines TNF-α, IL-6, & IL- 1β levels (Fig. 2). The primary inflammatory mediator (NO), was produced in large amounts by LPS-induced RAW 264.7 cells[42]. These pro-inflammatory cytokines, TNF-α, IL-6, and IL-1, are important factors in an inflammatory reaction and the beginning of inflammation[43]. We observed that *L. fermentum* MTCC 25515 was able to reduce excessive production of pro inflammatory cytokines. Our finding was in line with the earlier reported studies[44,45]. They evaluated the anti-inflammatory potential of bacteria on RAW 264.7 cells. These findings suggest that *L. fermentum* MTCC 25515 may possess anti-inflammatory properties, as it reduced the production of pro-inflammatory cytokines.

Conclusions

Lactobacilli from fermented rice beverage *Limosilactobacillus fermentum* MTCC 25515 and *Lacticaseibacillus rhamnosus* M9 remained viable under simulated gastrointestinal environment and had considerable adherence and aggregation ability. Both the lactobacilli displayed antimicrobial properties against indicator organisms and produced short chain fatty acids in skim milk. However, they were negative for bile salt hydrolase activity. Considering the safety criteria for these potential probiotic lactobacilli, both were found negative for biogenic amines production, gelatinase, lecithinase and hemolyticactivity, and showed moderate to low antibiotic resistance. The *L. fermentum* MTCC 25515 also exhibited anti-inflammatory activity when exposed to RAW 264.7 cells treated with lipopolysaccharide. Both the potential probiotic strains can be further investigated in animal and clinical trials.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_23(08)(2024)787-798_SupplData.pdf

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Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

MRK: Conceptualization, biochemical and microbial analysis, writing, drafting, reviewing the manuscript; RM, MB, KKK: Biochemical analysis and writing the manuscript; YV: Writing, editing and reviewing the manuscript; SH: Conceptualisation, editing, reviewing the manuscript

Data Availability

The data is available from the corresponding author upon reasonable request.

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