Current Research in Food Science 6 (2023) 100507

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

In vitro safety assessment of electrohydrodynamically encapsulated *Lactiplantibacillus plantarum* CRD7 and *Lacticaseibacillus rhamnosus* CRD11 for probiotics use

Vinay Venkatesh Varada [a], Divya Panneerselvam [b], Heartwin A. Pushpadass [b], Rashmi Hogarehalli Mallapa [c], Chand Ram [c], Sachin Kumar [a,*]

[a] Rumen Biotechnology Lab., Animal Nutrition Division, ICAR-National Dairy Research Institute, Karnal, 132001, Haryana, India
[b] Dairy Engineering Section, ICAR-National Dairy Research Institute, Southern Regional Station, Bengaluru, India
[c] Dairy Microbiology Division, ICAR-National Dairy Research Institute, Karnal, 132001, Haryana, India

ARTICLE INFO

Keywords:
Lactobacillus species
Probiotics
Safety assessment
Antibiotic resistance
Virulence genes

ABSTRACT

The current study aimed to validate the safety of electrohydrodynamically encapsulated *Lactiplantibacillus plantarum* CRD7 and *Lacticaseibacillus rhamnosus* CRD11 in accordance with guidelines of FAO/WHO and ICMR/DBT. *In vitro* assays such as mucin degradation, hemolysis of blood cells, antimicrobial susceptibility pattern, possession of virulence factors, biogenic amine, and ammonia production were assessed. In results, the cross-streak and co-culture techniques revealed that CRD7 and CRD11 were compatible *in vitro*. Upon visual inspection through scanning electron and fluorescence microscopy, the integrity of bacterial cell membrane was confirmed even after the encapsulation process. CRD7 and CRD11 were non-hemolytic and showed negative responses to gelatinase, urease, and DNase activities. Non-mucinolytic activity of CRD7 and CRD11 was verified by measuring cell growth rate ($p < 0.05$) in different modified media followed by SDS-PAGE. High-performance liquid chromatography analysis revealed that both the strains did not produce biogenic amines (putrescine, cadaverine, histamine, and tyramine). Neither of the *Lactobacillus* strains produced ammonia after growing in BHI broth for 5 days at 37 °C. L-lactate production was highest ($p < 0.05$) in CRD11 (8.83 g/L), followed by CRD7 (8.16 g/L), whereas the lowest ($p < 0.05$) D-lactate was registered for encapsulated CRD11 (0.33 g/L) and CRD7 (0.49 g/L). The antibiogram profile determined through minimum inhibitory concentration showed that CRD7 and CRD11 were sensitive to key antibiotics suggested by EFSA except for gentamycin and kanamycin. PCR data on virulence genes demonstrated that both strains were safe for probiotic use. Moreover, CRD7 and CRD11 strains caused insignificant ($p > 0.05$) changes in the cell viability of Caco-2 cells as estimated by MTT (98.94–99.50%) and NR uptake (95.42–97.03%) assays and showed sensitivity to human serum. According to the results of these evaluated attributes, it is concluded that *L. plantarum* CRD7 and *L. rhamnosus* CRD11 are safe, non-toxic to human epithelial cells, and thus may be potentially suitable for various food/feed applications.

1. Introduction

Probiotics are live microorganisms that have been shown to provide a wide range of health benefits to the target host, such as colonisation resistance against pathobionts in the gastrointestinal tract (GIT), effectively establishing healthy intestinal microflora, enhancing digestion, and improving immune function (Kumar et al., 2017a; Markowiak and Slizewska, 2017). Antibiotics, on the other hand, are used as preventive or curative measures against bacterial infections (Pattanaik et al., 2022). But the unscrupulous and uncontrolled application of antibiotics has given rise to the issue of antimicrobial resistance (AMR) that poses a serious threat to the health and longevity of both humans and animals (Kumar et al., 2021; Pradhan et al., 2020; Umar et al., 2020). The gut is considered the epicenter of AMR. The horizontal transfer of clinically relevant AMR genes and virulence factors from high-risk pathogens to the native gut microbiome turns harmless bacteria into virulent microbes and makes it difficult to eradicate them (Carlet, 2012; Das et al., 2020b). For instance, previous research has shown that certain strains

* Corresponding author.
E-mail address: arensachin@gmail.com (S. Kumar).

https://doi.org/10.1016/j.crfs.2023.100507
Received 3 March 2023; Received in revised form 18 April 2023; Accepted 21 April 2023
Available online 22 April 2023

can survive antibiotic treatment due to resistance genes transferred via plasmids in the guts of humans and animals, resulting in the spread of AMR (Fouhy et al., 2012). Thus, the use of live bacterial species as antibiotic alternatives has become increasingly important over time. In this context, *Lactobacillus* species have emerged as a promising, health-promoting group of beneficial microbes. Lactic acid bacteria (LAB) are shown to produce various enzymes and metabolites (reuterin, bacteriocin, organic acids, etc.), which play key roles in different industrial applications (Markowiak and Slizewska, 2017). For example, strains with high β-galactosidase, peptidase, and hydrolase activities may be useful in the production of commercially available lactose-free fermented milk products and cheese making and support antioxidant effects in various food products (Saqib et al., 2017; Fugaban et al., 2021).

In recent years, probiotics have become increasingly popular among consumers owing to their numerous health benefits. There are ample documented evidences describing the use of LAB as probiotics. *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* are commonly isolated from fermented foods and known to possess versatile properties that promote gut health in humans (Das et al., 2020a; Umar et al., 2020) and other species such as poultry (Reuben et al., 2019), swine (Liu et al., 2022), canines (Kumar et al., 2017b, 2021), and calves (Singh et al., 2021a; Varada et al., 2022a). Previous *in vivo* studies demonstrated that administration of *Lactobacillus* effectively alleviates diarrhea, strengthens the gut barrier function, promotes intestinal digestion, reduces pro-inflammatory cytokines, and exhibit hypocholesterolemic and hypoglycaemic effects (Russo et al., 2020; Thumu and Halami, 2020; Varada et al., 2022b). However, their usage accompanies major safety concerns because some LAB strains have been linked to septicaemia, urinary tract infections, endocarditis, liver abscess, and sepsis in patients (Vesterlund et al., 2007; Yakabe et al., 2009; Pradhan et al., 2020). As a result, before incorporating a new strain into a product for human use, the efficacy, effectiveness, and preclinical safety should be assessed on a case-by-case basis (FAO/WHO, 2006; Ganguly et al., 2011).

Currently, a variety of commercial probiotic microorganisms are being developed and marketed without complying with labeling laws and regulations prescribed by the European Commission (EC). Highlighting this concern, Huys et al. (2013) recommended in FAO guidelines for food industry probiotic application as follows: (1) establishing microorganism identity (determination of bacterial genus, species, and strain); (2) *in vitro* assays to screen potential probiotic strains (ability to tolerate acid and bile, potent antimicrobial activity, etc.); (3) evaluation of safety: requirement of valid evidence that a particular probiotic strain is safe and poses no risk in its delivery form; and (4) *in vivo* efficacy studies for the substantiation of the claimed health effects in the appropriate and validated animal models, prior to human trials. In addition, fulfilling the "Qualified Presumption of Safety" (QPS) concept standards given by EFSA is necessary for edible microorganisms used in food and feed formulations (EFSA, 2007). The American Gastroenterological Association (AGA) limits the use of probiotics for most of the digestive disorders (Crohn's, ulcerative colitis, or irritable bowel syndrome) due to a clear lack of evidence to support their efficacy (Su et al., 2020). Therefore, systematic research is mandatory in order to confirm the full safety attributes of a candidate probiotic strain before LAB is mass-produced for commercial and academic use.

The FAO/WHO (2006) recommended that probiotic products should contain 10^7–10^8 colony forming units/g of live bacterial cells at the time of consumption to realise their intended health benefits (Parsana et al., 2023). Undeniably, designing an effective delivery system is an essential criterion for enhancing probiotic efficacy (Coelho-Rocha et al., 2018). However, previous studies have reported that some commercially available and traditional dairy probiotic products suffered substantial reductions in their viability compared to the number of administered bacteria in gastric fluids and during storage (Dodoo et al., 2017; Duman and Karadag, 2021; Ma et al., 2021). To circumvent this drawback, the electrohydrodynamic technique is proposed as a novel method of encapsulating bacteria with a prebiotic matrix that allows probiotics to colonise in the human gut with greater viability (Moayyedi et al., 2018). So far, there are only a few studies available that used a prebiotic matrix for probiotic encapsulation using the electrohydrodynamic technique in comparison to other encapsulation methods.

Although the numerous studies on the application of *Lactobacillus* strains are promising and encourage continuous innovation for the applications of beneficial LAB strains, the debate on their safety as probiotics still continues. Hence, this study aimed to evaluate the safety and viability of electrohydrodynamically encapsulated *Lactiplantibacillus plantarum* CRD7 and *Lacticaseibacillus rhamnosus* CRD11 for probiotic usage.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and coexistence test

The probiotic strains used in the present investigation were *Lactiplantibacillus plantarum* CRD7 (GenBank Accession No. - KJ769142) and *Lacticaseibacillus rhamnosus* CRD11 (GenBank Accession No. - KJ769145). The details of all the other cultures used for safety evaluation in the present study are listed in Supplementary Table 1. Probiotic strains from glycerol stocks were grown in de Mann Rogosa and Sharpe broth (MRS; HiMedia Laboratories, Mumbai, India; Cat. No. GM369-500G) and incubated at 37 °C for 24 h. The active cultures were then sub cultured 3–4 times in MRS broth before use. The identity and purity of both the test cultures were confirmed by performing phenotypic tests (Gram staining, negative staining, and the catalase test) and genus-specific PCR. The compatibility assay between the selected LAB strains was performed by co-culture and cross streak assay according to the method of Varada et al. (2022a). Briefly, both the probiotic cultures were spotted independently and adjacent to each other in the co-culture technique, while they were streaked across each other and perpendicularly in the cross-streak assay using a 1 μL sterile loop on MRS agar (1.5% w/v; HiMedia Laboratories, India; Cat. No. GM641-500G) plates. The cultured plates were anaerobically incubated for 2 days at 37 °C to check the synergism or antagonism between the two lactobacilli strains.

2.2. Encapsulation of probiotic strains, scanning electron microscopy (SEM), and cell membrane integrity

Encapsulation of *L. plantarum* CRD7 and *L. rhamnosus* CRD11 was done by following the method described by Ma et al. (2021) with some modifications. The feed solution was prepared by dissolving probiotics (CRD7 or CRD11, 20% w/v), polymer (pullulan, 14% w/v; Kumar Organic Products Ltd., Bangalore, India), prebiotic (inulin, 20% w/v; MP Biomedicals, USA), and lyoprotectant (trehalose, 10% w/v; HiMedia Laboratories, Mumbai, India) in sterilised distilled water and thoroughly mixed using magnetic stirrer (Model-C-MAG HS7, IKA Werke GmbH & Co.KG, Staufen, Germany) at 550 rpm for 45 min. This solution was fed to an electrospinning machine (Model-H/VPD40CH, Royal Enterprises, Chennai, India) for the microcapsule preparation. The microencapsulated probiotics were collected and transferred into Eppendorf vials for further analysis.

The surface morphology and microstructure characteristics of encapsulated *L. plantarum* CRD7 and *L. rhamnosus* CRD11 and blank nanofibers were determined by field emission gun scanning electron microscope (Model-GEMINI Ultra 55, Zeiss, Jena, Germany). The commercial kit (LIVE/DEAD® BacLight™, Bacterial Viability Kit, Molecular Probes, Invitrogen, USA) was used to test the cell membrane integrity of encapsulated and non-encapsulated forms of *L. plantarum* CRD7 and *L. rhamnosus* CRD11. According to the manufacturer's protocol, a bacterial suspension was prepared and mixed with the dye mixture provided in the kit (ratio of 1000:3 μL). Then, 05 μL of the stained bacterial suspension was loaded on a clean slide, covered with a square coverslip, and observed in a fluorescence microscope (Olympus BX51, Feasterville,

PA, USA). Each experiment was performed in triplicate, and ten different microscopy fields were examined for each independent experiment, respectively.

2.3. Hemolytic assay

Hemolytic activity of both the selected probiotic strains was performed following the method of Balamurugan et al. (2014). Briefly, overnight-grown *L. plantarum* CRD7 and *L. rhamnosus* CRD11 were spot-plated separately (each strain, 5 μL) on blood agar base (HiMedia, India; Cat. No. M073-500G) plate surface supplemented with 5% (v/v) defibrinized sheep blood and incubated for 48 h at 37 °C. The hemolytic activities of the cultures were interpreted based on the different zones of hemolysis. Beta hemolytic activity (β) is indicated by the presence of clear yellow zones surrounding the bacterial colonies. While a greenish to brown zone around the colonies was considered alpha hemolysis (α), and no clear zone around the colonies showed non-hemolytic or gamma hemolysis (γ). *Staphylococcus aureus* ATCC9144 was used as a positive reference strain that exhibited complete hemolysis (β hemolysis) and *Lactobacillus rhamnosus* NCDC347 as a negative control (γ hemolysis).

2.4. Gelatinase, urease, and DNase activity

The gelatinase enzyme production activity was examined using overnight grown lactobacilli cultures in MRS broth at 37 °C. Each strain (100 μL) was inoculated into BHI tubes (10 mL) containing 4% gelatin and incubated for 48 h at 37 °C, followed by cooling (30 min at 4 °C). The culture tube was tilted to observe gelatin liquefaction. The retention of liquid medium indicated a positive result for gelatinase activity due to hydrolysed gelatin (Fugaban et al., 2021). The urease enzyme production was determined by inoculating strains in urea agar medium (Christenson's agar; HiMedia, India; Cat. No. M112-100G) on plates with phenol red as the indicator. Positive urease activity is indicated by a pink ring around the colonies (Bhagwat and Annapure, 2019). For gelatinase and urease experiments, faecal flora harvested from healthy adult males and *Proteus vulgaris* NCDC73 were used as positive controls, while *L. rhamnosus* NCDC 347 was used as a negative control.

DNase activity was done adopting the method of Rastogi et al. (2020). The cultures were spot-inoculated (10 μl) on the surface of DNase agar medium (HiMedia, India; Cat. No. M1419-100G) and incubated for 3 days at 37 °C. The plates were flooded with 1 M HCl for 10 min. Any clear zone around the colony represented DNase activity. The positive control bacteria was *Staphylococcus aureus* ATCC9144, and *E. coli* ATCC25922 was used as the negative control.

2.5. Mucin degradation assay

Mucin degradation assay of the CRD7 and CRD11 strains was examined using the following three standard methods: 1) growth in liquid medium, 2) degradation assay in a petri dish, and 3) SDS-PAGE analysis of degraded mucin residues as reported previously (Zhou et al., 2001; Pradhan et al., 2019). The mucin from porcine stomach, Type III (HGM; Sigma-Aldrich, USA) was used after purification in all mucinolytic activity. The MRS basal media were prepared with four different carbon substrates (0.5% (w/v) mucin, 1% mucin (w/v), 0.5% (w/v) glucose, and 1% glucose (w/v)) and without a carbon source as a negative control. The respective cultures (100 μL) were inoculated into each of the above-given four MRS broth media (10 mL) and incubated at 37 °C for 48 h. The absorbance of the samples was measured at 600 nm for the determination of bacterial growth at 12, 24, 36, and 48 h of incubation. The absorbance of basal MRS media was treated as blank. Faecal flora was used as a positive control, and the autoclaved faecal sample (121 °C, 20 min) was used as a negative control. Each experiment was performed in triplicate, and the results were presented as mean ± standard deviation (SD).

Mucin degradation assay in a petri dish was assessed according to the protocol given by Abe et al. (2010). Briefly, agar (1.5% w/v) plate media containing four different carbon sources as mentioned above were prepared for the assay. An aliquot (10 μL) of each of the test cultures grown in the respective media was spot-inoculated on the surface of the mentioned dried agar plates and incubated for 3 days at 37 °C. After the incubation period, amido black (0.1% amido black in 3.5 M acetic acid) was poured on the plate's surface and then discoloured with acetic acid (1.25 M) after 30 min. Any zone of mucin lysis (clear halo) around the colony indicated positive mucin degradation activity.

The basal medium of test cultures was used in SDS-PAGE (12.5%) for further confirmation (Pradhan et al., 2019). At the end of incubation (37 °C for 18 h), the cultures were centrifuged (10,000 g, 4 °C, 30 min) to collect cell-free supernatant fluid. The supernatant was vortexed and centrifuged again after being mixed with 15 mL of chilled ethanol (1.5 times the amount of supernatant). The harvested pellet was suspended in 0.5 ml of Tris–HCl buffer (10 mM) and used as the SDS-PAGE sample. Gels were stained with both periodic acid-Schiff (PAS, GelCode Glycoprotein Staining Kit, Thermo Scientific, IL, USA) and silver stain (Pierce™ Silver Stain Kit, Thermo Scientific, IL, USA) for glycoprotein pattern. Any *de novo* band with a smaller molecular weight compared with the negative control (autoclaved faecal flora) was defined as positive mucin degradation.

2.6. Biogenic amine production

The amino acid decarboxylase enzyme production ability of probiotic strains was evaluated by qualitative and quantitative chemical analysis of the biogenic amine (BA) potentially formed in the fermenting broth. An induction assay was performed on previously grown test cultures and a positive control by subculturing in MRS and brain heart infusion (BHI) broth (HiMedia, India; Cat. No. M210-500G), respectively, supplemented with 1% (w/v) of respective individual amino acid precursors (histidine monohydrochloride, ornithine monohydrochloride, lysine monohydrochloride, and tyrosine; Sigma-Aldrich, St. Louis, MO, USA) and 0.005% of pyridoxal-5-phosphate (Bover-Cid and Holzapfel, 1999). The broth tubes were checked for a final pH change caused by the production of the more alkaline biogenic amine. The final batch of actively growing cultures was also streaked on decarboxylase medium agar plates (1.5% w/v) containing corresponding amino acid precursors and without amino acids (as a control) and incubated at 37 °C for 72 h. At the end of incubation, purplish discoloration around the colonies indicated biogenic amine positivity due to a pH shift in response of the indicator. Reference strains in this assay were *Enterococcus faecalis* NCDC114 (positive control) and *L. rhamnosus* NCDC347 (negative control).

The quantitative determination of BA production was carried out using high-performance liquid chromatography (HPLC) according to the method described by Kim et al. (2018). For standard preparation, four biogenic amines were purchased from Sigma-Aldrich, St. Louis, MO, USA (putrescine, Cat. No. 51799; cadaverine, Cat. No. 33211; histamine, Cat. No. H7125; and tyramine, Cat. No. T90344). The detailed sample extraction procedure from cultured media and analysis protocol were consistent with the previously published report (Alayande et al., 2020). Briefly, 50 mL of the bacterial culture was centrifuged at 10,000 *g* for 10 min at 10 °C. Biogenic amines were extracted from 5 ml samples with 25 ml of 0.4 M perchloric acid and transferred to a screw-capped vial. The mixture was then vortexed with 1000 μL of each crude extract, 200 μL of 2M sodium hydroxide, 10 μL of 1, 7-diaminoheptane (internal standard, 100 mg/L), 1000 μL of dansyl chloride (10 mg/mL in acetone), and 300 μL of saturated sodium carbonate until homogenization.The thoroughly mixed suspension was incubated in a dark water bath at 70 °C for 30 min. Subsequently, 100 μL of ammonium hydroxide (30% w/v) was added to each sample to remove excess dansyl chloride. After collecting the aqueous layer, the volume was adjusted to 5 mL with acetonitrile. The reconstituted sample and standard were filtered through a polytetrafluoroethylene membrane filter (0.45 μm), and kept at −20 °C prior

to HPLC analysis. The HPLC was performed with the following conditions provided in Supplementary Table 2.

2.7. Antibiotic susceptibility test

The antibiotic susceptibility assay of both probiotic strains was evaluated on Muller-Hinton agar (MHA; HiMedia, India; Cat. No. M173-500G) using the disc diffusion assay according to the protocol of Charteris et al. (1998). A panel of 12 common antibiotic discs of different classes (all purchased from HiMedia, India) were used: gentamicin (GEN; 10 μg), kanamycin (K; 30 μg), streptomycin (S; 10 μg), nalidixic acid (NA; 30 μg), tylosine (TL; 15 μg), chloramphenicol (C; 30 μg), ampicillin (AMP; 10 μg), tetracycline (TE; 30 μg), erythromycin (E; 15 μg), clindamycin (CD; 2 μg), trimethoprim (TR; 5 μg), and vancomycin (VA; 30 μg), all selected following the European Food Safety Authority (EFSA) recommendations to assess bacterial resistance to antimicrobials of human and veterinary importance (EFSA, 2012). After incubation at 37 °C for 24 h, inhibition zone diameters were measured with an electronic digital vernier calliper (measuring range of 0–150 mm; accuracy of 0.02 mm) and recorded according to Clinical and Laboratory Standardization (CLSI, 2015) criteria. In addition, MICs (minimum inhibitory concentrations) were tested for the following antibiotics within the range of concentrations given in parentheses (mg/L): ampicillin (64–0.125), vancomycin (128–0.25), gentamicin (512-1), kanamycin (2048-4), streptomycin (2048-4), erythromycin (32–0.06), clindamycin (128–0.25), tetracycline (256–0.5), and chloramphenicol (128–0.25). MICs were determined through the micro-broth dilution technique by cultivating CRD7 and CRD11 strains in MRS liquid medium at 37 °C for 48 h. The turbidity of bacterial cell suspensions was adjusted to 0.5 MacFarland unit to ensure uniformity. The microdilution plates were prepared with a series of twofold dilutions of antibiotics at various final concentrations in a 96-well microplate (Supplementary Table 3). A 100 μL diluted bacterial suspension was added to each well, and the final concentration of the bacteria was 10^5 CFU/ml. Bacteria-free MRS medium (100 μL) was added to one of the 12 columns of the plate as a negative control (blank). The plate was incubated at 37 °C for 24 h and bacterial growth was read by a microplate reader at 630 nm. The MIC value was determined as the minimum antibiotic concentration of the wells with complete bacterial inhibition in comparison with an antibiotic-free control well. The cut-off values for bacteria were based on the break points as prescribed by the EFSA guidelines. Both experiments were performed in triplicate.

2.8. Screening for the presence of potential virulence genes

According to the manufacturer's protocol, overnight grown CRD7 and CRD11 in MRS at 37 °C were used for the DNA isolation using the commercial kit (QIAamp® DNA Microbiome Kit, QIAGEN Inc., Valencia, CA, USA; Cat. No. 51704). DNA purity and quantity were determined using a Nanodrop spectrophotometer (Epoch™ 2, Vermont, USA). Bacterial DNA was screened for the presence of potential virulence genes by PCR according to the recommendation of the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Rychen et al., 2017). The PCR conditions and oligonucleotide primer sequences are mentioned in Supplementary Table 4. The detailed PCR reactions were consistent with the previously published report (Hussein et al., 2020). The PCR products were analyzed on a 1.5% agarose gel and visualized using GENVIEW™ gel documenter (Genetix, Asia) to confirm the DNA fragment size.

2.9. In vitro cytotoxicity assay

In vitro cytotoxicity of *L. plantarum* CRD7 and *L. rhamnosus* CRD11 was checked using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) dye reduction and neutral red (NR) uptake assays. The tetrazolium dye reduction experiment was based on cellular viability, considering that viable cells metabolize tetrazolium colourless salt to blue formazan crystals in mitochondria. The neutral red uptake assay was performed to evaluate the accumulation of neutral red dye in lysosomes of viable cells. Caco-2 cells were seeded (~1×10^5 cells/wall) in 6 well tissue culture plates to form cell monolayers (80–90% confluency) before being incubated at 37 °C for 15 days in a humidified CO_2 incubator (95% air and 5% CO_2). The test strains CRD7 and CRD11 (10^9 CFU/mL) solubilized in DMEM media were added to the wells in triplicates and incubated for 24 h. Later, the grown cells were either used for MTT dye reduction (Hong et al., 2008) or neutral red (NR) uptake assays (Bhat et al., 2019) for cytotoxicity measurement. DMEM-treated cells without bacteria were used as a control. Finally, the cell viability was calculated using the following equation:

$$\text{Viability percentage} = (\text{Test optical density/Control optical density}) \times 100$$

2.10. Resistance to serum

The serum resistance assay was performed as suggested by Vesterlund et al. (2007). Blood samples (approximately 20 mL) were collected into evacuated tubes (BD Vacutainer®) without anticoagulant from a healthy adult donor and then allowed to clot to harvest sera. The serum was thawed (at 56 °C for 30 min) to inactivate the complement components. The aliquots were pooled and stored in a deep freezer at −20 °C until further analysis. The overnight-grown culture was centrifuged (10,000 g for 10 min) to harvest cell pellets, washed twice with phosphate-buffered saline (PBS; pH 7.2), and re-suspended in the same buffer. Then the optical density (OD) was adjusted to 0.5 ± 0.01 at 600 nm to obtain approximately 10^7–10^8 CFU/mL number of bacteria. The bacterial suspension (200 μL) was mixed with heat-inactivated serum (800 μL), serum, or PBS (a negative control) and incubated at 37 °C for 90 min. The reaction was then stopped by placing it on ice packs (4 °C) for 10 min and performing serial dilutions in PBS. One milliliter of sample dilutions were plated on suitable media and incubated for 48 h at 37 °C. Faecal flora was included as a positive control. Each experiment was performed in triplicate.

2.11. D/L-lactic acid production test

D- and L-lactic acid produced by *L. plantarum* CRD7 and *L. rhamnosus* CRD11 strains was quantified using a commercially available kit (Megazyme® International, Bray, Ireland). The test was performed according to the manufacturer's protocol. Briefly, the probiotic strains were grown in MRS broth for 24 h at 37 °C, followed by centrifugation (10,000 *g* for 10 min) to obtain cell-free supernatant (CFS). The CFS (0.1 mL) was mixed with 1.5 mL of H_2O, 0.5 mL of buffer solution (pH 10.0), 0.1 mL of NAD^+ solution, and 0.02 mL of glutamate-pyruvate transaminase (GPT) and incubated at room temperature. The absorbance (A_1) after 3 min was noted at 340 nm. Subsequently, 0.02 mL of lactate dehydrogenase (LDH; 2000 U/mL) was added to the reaction mixture. The entire content was mixed, and the absorbance (A_2) was measured after 5 min at 340 nm. The absorbance of D-lactate was measured at 340 nm until the reaction stopped. The results were calculated using a spreadsheet provided by the manufacturer of the D-/L-lactic acid assay kit (http://www.megazyme.com). The lactic acid produced by each *Lactobacillus* strain was interpreted in proportion, indicating the quantity of both D- and L-lactic acid. *Leuconostoc mesenteroides* NCDC633 was used as the reference positive culture for D-lactic acid production, whereas *E. faecalis* NCDC114 was used for L-lactic acid production.

$$c = \left(\frac{V \times MW}{\in \times d \times v}\right) \times \Delta A$$

where, c = Concentration of D-/L-lactic acid (g/L), V = Final volume (mL) (2.24), MW = Molecular weight of D-/L-lactic acid (g/mol) (90.1),

ε = Extinction co-efficient of NADH at 340 nm (6300), d = Light path (cm) (1), v = sample volume (mL) (1), $\Delta A = A_2 - A_1$

2.12. Ammonia production test

An ammonia production experiment with *Lactobacillus* strains was based on a previously described method (Kim et al., 2018). The test cultures were grown in BHI broth at 37 °C for 5 days. The ammonia produced was estimated by a catalyzed indophenol reaction involving two reagents: solution A (phenol with sodium nitro ferricyanide dehydrates) and solution B (sodium hydroxide and sodium hypochlorite), as per the method of Chaney and Marbach (1962). The concentration of ammonia was found from a standard curve generated by processing a series of standards. *Pseudomonas aeruginosa* NCDC105 and faecal flora were used as positive controls, and an untreated medium was used as a negative control.

2.13. Statistical analysis

All the experiments were performed in triplicate, and the obtained data in this study were presented as mean ± standard deviation (SD). The result of mucin degradation assay (growth in liquid media) was subjected to a two-ways analysis of variance (ANOVA). One-way ANOVA was applied to compare the statistical differences among different cultures using SPSS 18.0 (SPSS Inc., version, Chicago, IL, USA) and was considered significant at $p \leq 0.05$. The graphs were developed using GraphPad Prism (version 8.01). The heat mapper web server (http://heatmapper.ca/) was used to plot correlation matrix. Heat map was created with ClustVis online software (https://biit.cs.ut.ee/clustvis/).

3. Results

3.1. Scanning electron microscopy, morphology, and cell membrane integrity

SEM was used to inspect the structure and surface morphology of blank nanofibers as well as encapsulated *L. plantarum* CRD7 and *L. rhamnosus* CRD11 (Fig. 1A, B, and 1C, respectively). On examination, the shapes of encapsulated CRD7 and CRD11 were found to be smooth surfaces with bacilli shape and an undamaged cellular appearance. On the other hand, blank nanofibers exhibited a uniform, smooth, and beadless structure. Bright field and fluorescence microscopic images of encapsulated and nonencapsulated *L. plantarum* CRD7 and *L. rhamnosus* are depicted in Fig. 1D, E, 1F, and 1G. Live bacteria (with intact cell membranes) were fluoresced green, whereas dead bacteria (with damaged cell membranes) were fluoresced red. The results demonstrated that the bacterial cell membrane integrity was not damaged by the electrohydrodynamic encapsulation technique, as was the case with active cultures of *L. plantarum* CRD7 and *L. rhamnosus* CRD11.

3.2. Coexistence test

In the present study, the strain compatibility test was performed with the aim of designing multi-strain probiotics due to the synergistic action of mixed bacterial cultures. Both the probiotic isolates *L. plantarum* CRD7 and *L. rhamnosus* CRD11 revealed their synergistic properties and complemented each other functionally *in vitro*, as depicted in Fig. 2A and B. Based on the obtained data and assuming that these additive effects could also occur in *in vivo*, both CRD7 and CRD11 were encapsulated using the electrohydrodynamic technique for food application.

3.3. Hemolytic assay

Hemolytic assay is an essential criterion for the potential use of the probiotic strains. CRD7 and CRD11 didn't exhibit β or α-hemolytic activities, and no change in colour was observed around the cell colonies in this study (Fig. 2C). While *Staphylococcus aureus* ATCC9144 (positive control) and *L. rhamnosus* NCDC 347 (negative control) showed β and γ hemolysis, respectively.

3.4. Gelatinase, urease, and DNase activity

Both the strains CRD7 and CRD11 showed negative results for gelatinase (Fig. 2D), urease (Fig. 2E), and DNase (Fig. 2F) activities. Positive controls included *Proteus vulgaris* NCDC73 and faecal flora for gelatinase and urease assays (Supplementary Figs. 1A and B), and *Staphylococcus aureus* ATCC9144 for DNase activity exhibited positive results. Where L. *rhamnosus* NCDC347 and *E. coli* ATCC25922 as negative controls didn't produce gelatinase, urease, or DNase enzymes, respectively.

3.5. Mucin degradation assay

Different modified media were used to test the ability of *Lactiplantibacillus plantarum* CRD7 and *Lacticaseibacillus rhamnosus* CRD11 to degrade gastrointestinal mucosa (Supplementary Table 5). According to Fig. 3A and B, the growth of CRD7 and CRD11 was actively induced ($p < 0.05$) in glucose (0.5% and 1%) medium when added as a carbon source. However, negligible growth was observed in either strain when mucin (0.5% and 1%, respectively) was used instead of glucose. In addition,

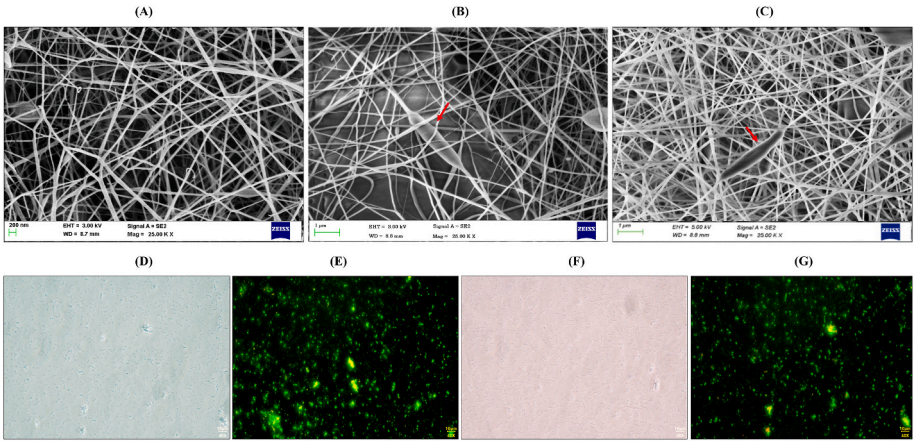

Fig. 1. Scanning electron microscopy (SEM) images (25,000 × magnification) of **(A)** blank nanofiber without bacteria, **(B)** *L. plantarum* CRD7 loaded nanofibers, and **(C)** *L. rhamnosus* CRD11 loaded nanofibers. Bright field microscopic image (40×magnification) of **(D)** non-encapsulated and **(F)** encapsulated forms of CRD7 and CRD11. Fluorescence microscopic images showing cell membrane integrity of **(E)** non-encapsulated and **(G)** encapsulated forms of CRD7 and CRD11.

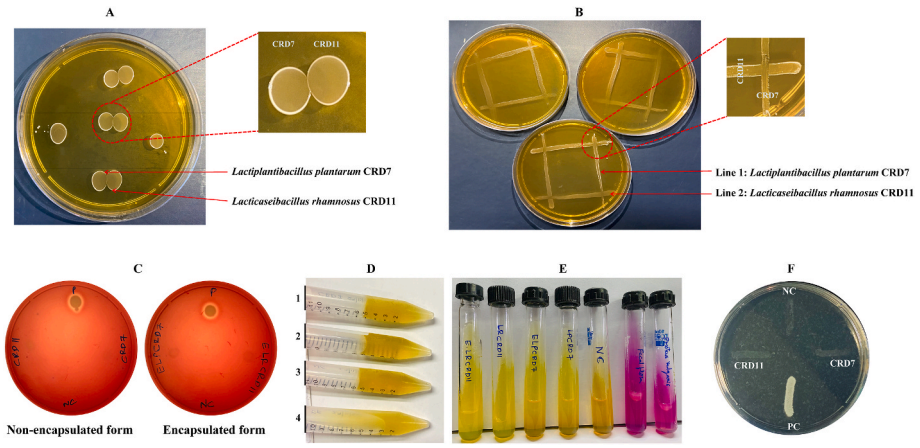

Fig. 2. Compatibility between *L. plantarum* CRD7 and *L. rhamnosus* CRD11 by **(A)** co-culture technique and **(B)** cross-streaked assay on the MRS agar plates. **(C)** Hemolytic assay. **(D)** Gelatinase activity. **1**, *L. plantarum* CRD7; **2**, *L. rhamnosus* CRD11; **3**, *L. rhamnosus* NCDC 347 (negative control); **4**, Faecal flora (positive control). **(E)** Urease activity. **(F)** DNase activity.

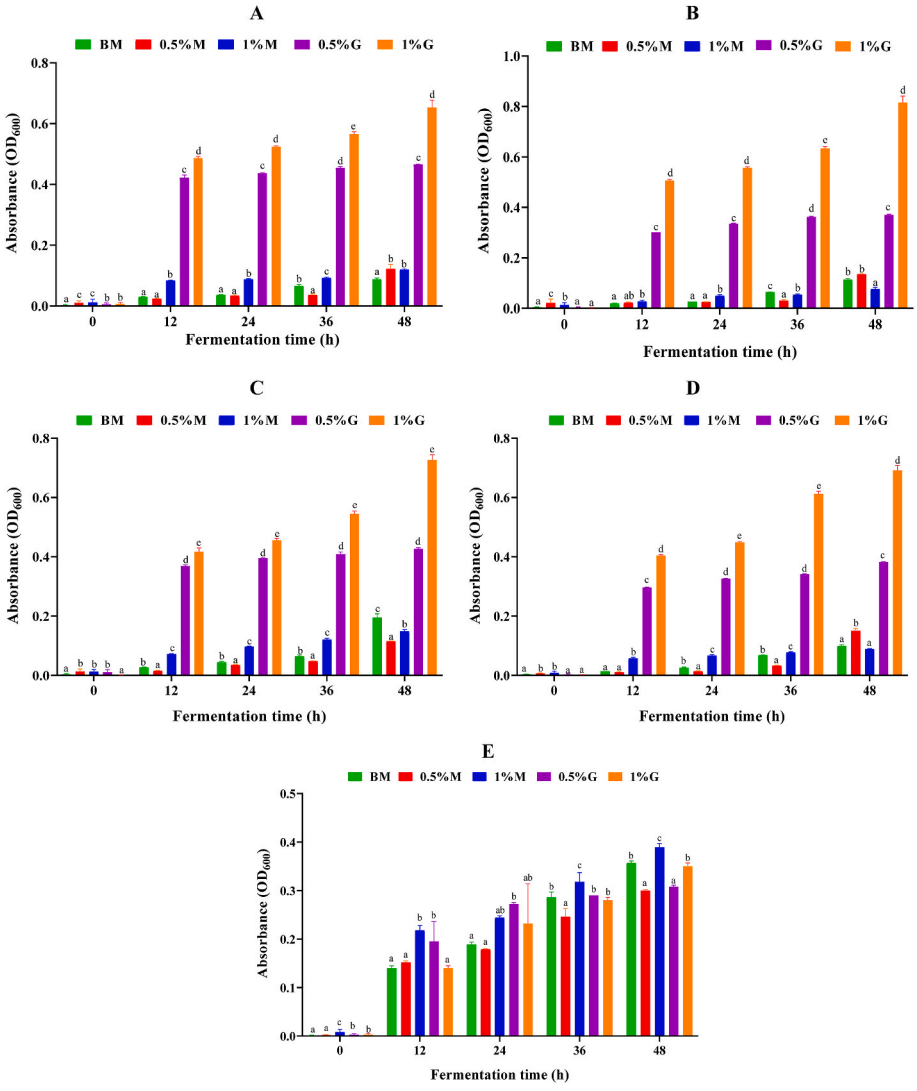

Fig. 3. The OD_{600} values of **A)** *L. rhamnosus* CRD11; **B)** *L. plantarum* CRD7; **C)** Encapsulated *L. rhamnosus* CRD11; **D)** Encapsulated *L. plantarum* CRD7; and **E)** Faecal flora were measured within 0, 12, 24, 36, and 48 h of fermentation culture. The five different media are basal medium (BM), BM with 0.5% hog mucin (0.5% M), BM with 1% hog mucin (1% M), BM with 0.5% glucose (0.5% G), and BM with 1% glucose (1% G). Superscripts (a, b, c, d, and e) represent the significant differences ($p < 0.05$) among different concentrations of media at different time interval (Analyzed by Two-way ANOVA using Duncan Post hoc test).

encapsulated CRD7 and CRD11 exhibited significant ($p < 0.05$) growth in glucose medium and were close to zero in mucin medium (Fig. 3C and D). Faecal flora (positive control) flourished in the carbon-free basal medium as well as in media containing glucose and mucin (Fig. 3E).

Similarly, the mucinolytic activity of test strains was performed in a petri dish stained with and without amido black (Supplementary

Figs. 2A, 2B, and 2C, 2D). Only faecal flora formed a clear lysis zone around the colony in all of the media when agar medium with four different substrates was used as a carbon source. However, no mucinolytic zone was observed for encapsulated and non-encapsulated forms of CRD7 and CRD11, while they grew abundantly in glucose-supplemented media. Also, the autoclaved faecal microbiota sample failed to produce a lysis zone around the inoculated spot on medium with mucin and glucose. Additionally, mucinolytic properties were also checked in the SDS-PAGE gel of the fermentates of both cultures. The PAS and silver staining of SDS-PAGE gels showed an intact band in the swim lane of CRD7 and CRD11, and no other small molecular bands were found (Supplementary Fig. 3A and 3B**)**. However, faecal flora displayed a faint smear of the glycoprotein residues when compared to heat-killed faecal flora. These findings indicated that faecal microbiota have a higher mucin-degrading capacity than the tested probiotic strains.

3.6. Biogenic amine production

The pH values of the decarboxylase media fermentates and biogenic amine content of the CRD7 and CRD11 cultures are depicted in Table 1. The results of the broth test for pH determination revealed that both the probiotic strains were negative for all the biogenic amino acid precursors (histidine, tyrosine, lysine, and ornithine) tested. As can be seen from Supplementary Figs. 4A, 4B, 4C, and 4D in petri dishes, both the probiotic strains were found negative for all four principal biogenic amines, which further supported their inability to produce decarboxylase enzymes. However, *Enterococcus faecalis* NCDC114 was used as a positive control and formed tyramine from tyrosine (Supplementary Fig. 4B). In addition, HPLC analysis carried out on the extracted biogenic amines revealed that both strains didn't produce putrescine, cadaverine, histamine, or tyramine during the fermentation process (Table 1).

3.7. Antibiotic susceptibility test

L. plantarum CRD7 *and L. rhamnosus* CRD11 were tested for antibiotic resistance against a panel of antibiotics, including those recommended by EFSA. As illustrated in Table 2, *L. plantarum* CRD7 and *L. rhamnosus* CRD11 showed susceptibility to the studied antibiotics following the zone diameter cutoff values prescribed by Charteris et al. (1998). However, both probiotic strains showed resistance to vancomycin and nalidixic acid (Supplementary Fig. 5). Additionally, CRD7 and CRD11 were sensitive to ampicillin, streptomycin, chloramphenicol, clindamycin, erythromycin, and tetracycline, except for gentamycin and kanamycin through the micro-broth dilution technique (MIC ranging from 0.25 to 32 mg/L) based on the breakpoints established by EFSA (2012) (Table 3). The MICs of *L. plantarum* CRD7 against tetracycline were equal to the cutoff value.

Table 1
Determination of biogenic amine production of different *Lactobacillus* strains.

Strain evaluated	Biogenic amino acid precursor used			
	Lysine	Histidine	Ornithine	Tyrosine
pH values of decarboxylase media fermentates				
L. plantarum CRD7	$5.18^{a} \pm 0.05$	$5.27^{b} \pm 0.04$	$5.20^{b} \pm 0.01$	$5.60^{c} \pm 0.09$
L. rhamnosus CRD11	$5.36^{b} \pm 0.03$	$5.22^{b} \pm 0.02$	$5.13^{a} \pm 0.03$	$5.08^{a} \pm 0.11$
Enterococcus faecalis NCDC114	$5.79^{c} \pm 0.07$	$5.92^{c} \pm 0.05$	$5.53^{c} \pm 0.07$	$6.36^{d} \pm 0.06$
L. rhamnosus NCDC347	$5.16^{a} \pm 0.02$	$5.13^{a} \pm 0.02$	$5.26^{b} \pm 0.02$	$5.44^{b} \pm 0.04$
HPLC method	**Biogenic amines detected**			
	Cadaverine (μg/mL)	**Histamine (μg/mL)**	**Putrescine (μg/mL)**	**Tyramine (μg/mL)**
L. plantarum CRD7	N/D	N/D	N/D	N/D
L. rhamnosus CRD11	N/D	N/D	N/D	N/D

N/D; not detected. HPLC; high-performance liquid chromatography.
Values are expressed as mean ± SD (n = 3) from three independent experiments. The values with superscripts a, b, c, d within the columns differed significantly ($p < 0.05$) among the different biogenic amino acid precursor used (Analyzed by One-way ANOVA using Duncan Post hoc test).

3.8. Screening for the presence of potential virulence genes

In this study, the ability of the *L. plantarum* CRD7 *and L. rhamnosus* CRD11 were screened for the presence of virulence and antibiotic resistance associated genes. The PCR-based screening test showed that both CRD7 and CRD11 strains were negative for the various target genes (*hyl*, *gelE*, *efaA*, *Ccf*, *cylA*, and *VanA*) assayed, thereby showing the strains to be safe for food application (Table 4). However, faecal flora DNA used as a positive control showed strong bands for the targeted potential virulence factor genes (Supplementary Fig. 6).

3.9. In vitro cytotoxicity assay

The toxicity of different forms (non-encapsulated and encapsulated) of *L. plantarum* CRD7 and *L. rhamnosus* CRD11 to the Caco-2 cell line was carried out using the spectrophotometric method to measure cell death. Our results depicted in Table 5 demonstrated that CRD7 and CRD11 strains caused insignificant changes ($p > 0.05$) in the cell viability of Caco-2 cells estimated by MTT and NR uptake assays even after 24 h of incubation.

3.10. Resistance to serum

L. plantarum CRD7 and *L. rhamnosus* CRD11 were found to be significantly sensitive ($p < 0.05$) to the bactericidal effect of human serum and grew well in comparison to the faecal flora (Fig. 4). However, the survivability percentage of CRD7 and CRD11 in heat-inactivated serum was significantly higher ($p < 0.05$) than the faecal flora.

3.11. D/L-lactic acid production test

The assessment of the ratio between the D (−) and L (+) isomers of lactic acid is considered one of the criteria for the evaluation of a potential probiotic candidate. In this study, probiotic strains produced less D (−) and more L (+) lactic acids ($p < 0.05$), which is one of the desirable attributes (Table 6). *Leuconostoc mesenteroides* NCDC633, on the other hand, was used as the reference positive culture and produced the most D (−) lactic acid (8.18 ± 0.28 g/L) ($p < 0.05$), whereas *E. faecalis* NCDC114 (the negative control) produced trace amounts of D (−) lactic acid (0.42 ± 0.10 g/L) and a major amount of L (+) lactic acid (7.70 ± 0.13 g/L) ($p < 0.05$) in the media fermentate.

3.12. Ammonia production test

The ammonia production of *L. plantarum* CRD7 and *L. rhamnosus* CRD11 was evaluated to verify the safety of these probiotic strains. In the present study, CRD7 and CRD11 cultures did not produce ammonia. In contrast, faecal flora and *Pseudomonas aeruginosa* NCDC105, which are considered harmful bacteria and used as positive controls in the current study, produced 6.92 ± 1.42 and 10.34 ± 0.65 μg/mL of ammonia ($p < 0.05$), respectively (Table 6**)**.

3.13. Correlation analysis and heatmap visualization

The correlation matrix was plotted to get good overview of the interrelation among the analyzed parameter variables with $r < 0.3$ as weak (blue colour); moderate as 0.3–0.7 (green colour), and >0.7 (yellow colour) as strongly correlated. (Fig. 5A). A heat map was

Table 2
Antimicrobial sensitivity profiles of *Lactobacillus* strains on the basis of Zone of Inhibition (ZI) in mm.

Antibiotics	Code name (Concentration, µg/disc)	*L. rhamnosus* CRD11	*L. plantarum* CRD7	Status	
				CRD11	CRD7
Gentamicin	GEN (10)	24.67 ± 3.06	26.33 ± 3.21	S	S
Kanamycin	K (30)	21.33 ± 3.21	13.00 ± 2.00	S	I
Streptomycin	S (10)	23.00 ± 2.65	20.67 ± 1.53	S	S
Nalidixic acid	NA (30)	0.00	0.00	R	R
Tylosine	TL (15)	34.33 ± 2.08	34.67 ± 1.53	S	S
Chloramphenicol	C (30)	26.00 ± 3.61	21.00 ± 3.46	S	S
Ampicillin	AMP (10)	34.67 ± 1.53	32.33 ± 2.52	S	S
Tetracycline	TE (30)	28.00 ± 3.61	22.33 ± 2.08	S	S
Erythromycin	E (15)	37.00 ± 3.00	35.67 ± 2.08	S	S
Clindamycin	CD (2)	38.33 ± 2.52	36.00 ± 2.65	S	S
Trimethoprim	TR (5)	32.00 ± 1.00	30.67 ± 2.52	S	S
Vancomycin	VA (30)	0.00	0.00	R	R
	Lowest Value			**Highest value**	

Table 3
Minimum Inhibitory Concentration (MIC) of *Lactobacillus* strains against antibiotics specified in the European Food Safety Authority (EFSA) guidelines.

Antibiotics	EFSA cut-off (mg/L)*		Experimental MIC	
	L. rhamnosus	*L. plantarum*	*L.rhamnosus* CRD11	*L.plantarum* CRD7
Ampicillin	4	2	2	2
Vancomycin	NR	NR	32	128
Gentamycin	16	16	32	64
Kanamycin	64	64	128	128
Streptomycin	32	NR	32	16
Erythromycin	1	1	0.25	0.5
Clindamycin	1	2	0.5	1
Tetracycline	8	32	1	32
Chloramphenicol	4	8	4	4
			S	R

NR,breakpoint not required;S, Susceptible; R, Resistant; EFSA, European Food Safety Authority

Values expressed are mean from three independent experiments
*Strains with MIC higher are considered resistant. Breakpoints are outlined in EFSA reports (EFSA, 2012).

generated to cluster and visualize multivariate data of probiotic strains using online web tool (ClustVis). Accordingly, four probiotic strains were grouped into two major clusters as presented in Fig. 5B.

4. Discussion

The present investigation was conducted to assess the safety of two *Lactobacillus* test strains through an *in vitro* approach. However, the majority of the probiotic bacteria are extremely sensitive and lose

Table 4
Molecular-based assay for the detection of various potential virulence and/or antibiotic resistance genes in *Lactobacillus* strains.

Target protein/enzyme	Target gene	Strains evaluated	
		L. rhamnosus CRD11	*L. plantarum* CRD7
Hyaluronidase	*hyl*	–	–
Endocarditis antigen	*efaA*	–	–
Sex pheromones, chemotactic for human leukocytes; facilitate conjugation	*Ccf*	–	–
Cytolysin	*cylA*	–	–
Gelatinase	*gelE*	–	–
Vancomycin resistance	*vanA*	–	–

− absence, + presence.

Table 5
The survival rates (%) of Caco-2 cells treated with *Lactobacillus* strains using MTT and Neutral Red assays.

Strain evaluated	Cell survival (%)	
	NR assay	MTT assay
L. plantarum CRD7	$95.42^{a} \pm 0.98$	$99.30^{a} \pm 0.59$
L. rhamnosus CRD11	$97.03^{a} \pm 2.50$	$98.99^{a} \pm 0.18$
Encapsulated *L. plantarum* CRD7	$96.79^{a} \pm 1.94$	$98.94^{a} \pm 0.66$
Encapsulated *L. rhamnosus* CRD11	$96.25^{a} \pm 2.78$	$99.50^{a} \pm 0.41$

Values are expressed as mean ± SD (n = 3) from three independent experiments. The values with superscripts (a) within the columns represents no significant ($p > 0.05$) differences among the cell cytotoxicity assays (Analyzed by One-way ANOVA using Duncan Post hoc test).

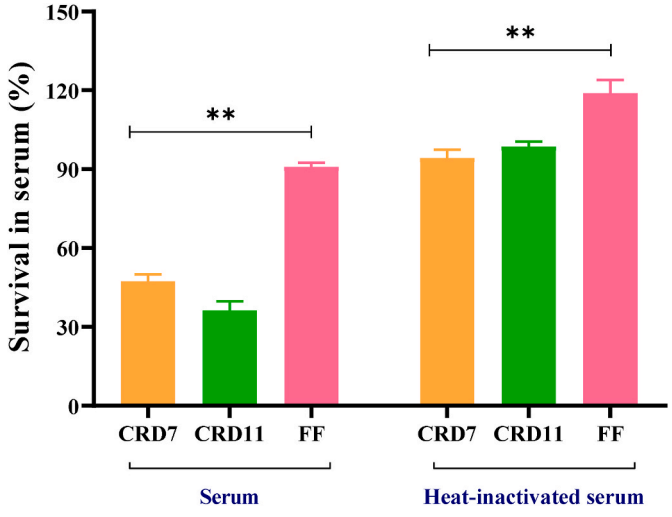

Fig. 4. Survival percentage of probiotic strains and faecal flora in human active serum and heat-inactivated serum. *L. plantarum* CRD7 (CRD7), *L. rhamnosus* CRD11 (CRD11), and faecal flora (FF). Each experiment was independently repeated three times. **$p < 0.01$ (Analyzed by One-way ANOVA using Duncan Post hoc test).

viability when exposed to unfavourable environmental conditions (Parsana et al., 2023). In this study, we used an electrohydrodynamic technique to encapsulate probiotics with a nano-prebiotic matrix to overcome the drawbacks of low survivability and to resist the unfavourable conditions of GIT for targeted delivery. Scanning electron and fluorescence microscopic images further confirmed the integrity of bacterial cell membranes even after the encapsulation process, therefore maintaining the survivability rates in GIT. Duman and Karadag (2021) and Ma et al. (2021) demonstrated an increase of up to 90% survival of

Table 6
D/L-lactic acid and ammonia production of different *Lactobacillus* strains.

Strain evaluated	D- lactic acid (g/L)	L- lactic acid (g/L)	Ammonia (μg/mL)
L. plantarum CRD7	$0.65^{bc} \pm 0.13$	$8.05^{c} \pm 0.14$	Negative
L. rhamnosus CRD11	$0.70^{c} \pm 0.38$	$8.78^{d} \pm 0.11$	Negative
Encapsulated *L. plantarum* CRD7	$0.49^{abc} \pm 0.03$	$8.16^{c} \pm 0.08$	Negative
Encapsulated *L. rhamnosus* CRD11	$0.33^{a} \pm 0.06$	$8.83^{d} \pm 0.13$	Negative
Leuconostoc mesenteroides NCDC633	$8.18^{d} \pm 0.28$	$0.06^{a} \pm 0.04$	NA
Enterococcus faecalis NCDC114	$0.42^{ab} \pm 0.10$	$7.70^{b} \pm 0.13$	NA
Faecal flora	NA	NA	$6.92^{a} \pm 1.42$
Pseudomonas aeruginosa NCDC105	NA	NA	$10.34^{b} \pm 0.65$

NA; not applicable. Values are expressed as mean ± SD (n = 3) from three independent experiments. The values with superscripts a, b, c, d within the columns differed significantly ($p < 0.05$) among the different metabolites produced (Analyzed by One-way ANOVA using Duncan Post hoc test).

encapsulated *L. fermentum* and *L. rhamnosus* with prebiotic nanofibers, respectively, under simulated GIT conditions and better viability during storage.

Hemolysis is a virulence factor demonstrated by pathogenic bacteria (e.g., streptococci and enterococci). Hemolysin, an immunogenic novel toxin, when it enters the blood, may dissolve antibodies and destroy red blood cells, resulting in anaemia and edema in the target host (Kim et al., 2018). *Lactobacillus* species are natural gut colonizers of humans and animals that have been widely used in functional foods. They may relatively pose a health threat as opportunistic pathogens, thereby threatening consumer health and food safety. As a result, assessing microbial hemolytic properties is an important criterion for potential probiotics. *L. plantarum* CRD7 and *L. rhamnosus* CRD11 did not show visible hemolysis on blood agar plates in this study, whereas *S. aureus* ATCC9144 (β hemolysis) showed hemolytic enzyme activity. Previous research with *L. plantarum* and *L. fermentum* found the α-hemolysis phenotype in human blood (Pradhan et al., 2019; Lee et al., 2022). Additionally, the production of various harmful enzymes (e.g., gelatinase, urease, and DNase) should be taken into consideration. Gelatinase is a protease enzyme that hydrolyzes bioactive peptides and has the capacity to breakdown collagen, casein, and haemoglobin, which are crucial components of both humans and animals' immune systems during systemic infection (Fugaban et al., 2021). Urease enzyme production activity was performed to rule out whether these strains release ammonia during urea substrate degradation (Bhagwat and Annapure, 2019). It is also recommended to check the deoxyribonuclease activity of bacteria to prove their safety. In this study, *Staphylococcus aureus* ATCC9144 showed positive DNase activity, while a negative result was registered for *E. coli* ATCC25922. Therefore, based on the results, it is found that both *Lactobacillus* strains are suitable for food and feed applications, especially as probiotic starter cultures in the dairy industry.

The intestinal mucosa is made up of glycoproteins, which serve as a first layer of physical barrier against invading pathogens and their toxic substances entering the blood stream of the host (Frenkel and Ribbeck, 2015). The intestine is covered by bilayer mucin, wherein the outer, wet surface is home to diverse microbiota but the inner layer is devoid of microbes. The mucinolytic property is exhibited by many high-risk pathogens (e.g., *Salmonella* spp., *Shigella* spp., *Helicobacter pylori*, *Staphylococcus aureus*, and *Vibrio cholerae*) as a virulence factor (Zhou et al., 2001). Previous research studies have demonstrated that bacterial translocation occurs due to the decomposition of gastric mucin, resulting in leaky gut and sepsis (Koyama et al., 2018). In such cases, the administration of probiotic strains was associated with the possibility of inducing endocarditis and bacteraemia in children (De Groote et al., 2005; Liong, 2008). Catabolic repression reduces mucinase synthesis

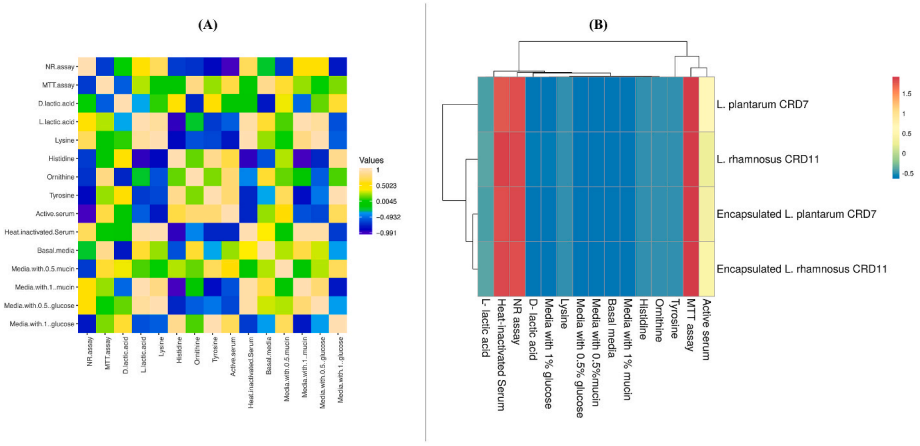

Fig. 5. A) Correlation matrix and **B)** heat map of *in vitro* safety assays such as cell cytotoxicity, biogenic amines, resistance to serum, lactic acid production, and mucin degradation activity of *Lactobacillus* strains.

when simple carbohydrates (e.g., fructose, glucose, and maltose) are introduced (Kim et al., 2018). Hence, different substrates were added as a carbon source in the MRS broth in the present study to obtain more accurate data because sometimes false negative results can be observed despite LAB strains' inability to produce mucinolytic enzymes (Lu et al., 2021). Mucin degradation activity was further confirmed by the petri dish method and SDS-PAGE clearly indicates that selected strains are incapable of damaging intestinal surfaces and lack translocational abilities. Consistent with this report, several other investigators also reported the absence of mucin degradation properties in potential probiotic strains (Singh et al., 2021b; Lu et al., 2021; Kumar et al., 2022).

Biogenic amines (BA) have been associated with toxic effects, including headaches, migraines, allergic reactions, stomach and intestinal ulcers, so their presence has major health effects on humans and animals (Jansen et al., 2003). According to Priyadarshani and Rakshit, (2011) and Fugaban et al. (2021), some LAB strains, especially lactobacilli and enterococci, were reported to exhibit decarboxylase activity, which reduces amino acids into BA in food production systems. Hence, we also examined our test strains (CRD7 and CRD11) for BA production as a component of an overall probiotic safety assessment and found them to be negative for all four major BAs (putrescine, cadaverine, histamine, and tyramine). Our results on BA production are similar to the previous reports (Pradhan et al., 2019; Lee et al., 2022), where the authors observed no decarboxylase enzyme activity.

EFSA (2012) prescribed that only non-resistant probiotic strains are to be used in human diets and animal feeds as a critical part of its safety qualification. According to breakpoints established by EFSA, both the tested strains were sensitive to clinically relevant antibiotics, except for vancomycin, gentamycin, and kanamycin. The disparity in antimicrobial sensitivity of *Lactobacillus* strains may be attributed to their taxonomic complexity, which is regarded as a genus and/or species characteristic (Singh et al., 2021b). These findings are in line with those of recently published reports (Gharbi et al., 2019; Lee et al., 2022). Since lactobacilli are recognized to be naturally resistant to vancomycin through a non-transferable resistance mechanism, a high rate of vancomycin resistance was reported (Singh et al., 2021b; Kumar et al., 2022). In addition, it has been noted that many *Lactobacillus* species are known to inherently possess resistance to gentamicin and kanamycin, which is a widespread phenomenon (Campedelli et al., 2019). The presence of D-alanine ligase-related enzymes and the lack of cytochrome-mediated electron transport in the cell wall of LAB render resistance to many antibiotics, especially aminoglycosides (Bernardeau et al., 2008). Pradhan et al. (2020) stated that the intrinsic resistance property is chromosomally encoded with a low risk for lateral transfer and has a low potential for posing a risk to non-pathogenic bacteria. Morever, no ARGs against virulence factors were detected in the PCR analysis of the two *Lactobacillus* strains. In most cases, these ARGs are naturally occurring, non-transferrable, and have been ascribed strain-specific characteristics (Umar et al., 2020). Furthermore, a molecular-based assay was used in this study for the detection of various potential virulence factors involved in translocation, evasion, and attachment. The screening for targeted genes was performed according to the EFSA guidelines recommended in FEEDAP. It was strongly suggested that screening for particular virulence genes should be carried out when LAB strains are found to be sensitive to the panel of antibiotics recommended by EFSA (Rychen et al., 2017). Thus, based on our findings, we speculate that selected *Lactobacillus* strains are free from potential virulence factors and antibiotic-resistance concerns.

The *in vitro* cytotoxicity tests (MTT and Neutral Red assays) are commonly employed for screening drugs and chemicals in toxicity studies of humans or animals (Eisenbrand et al., 2002). In this study, treatment of *L. plantarum* CRD7 and *L. rhamnosus* CRD11 with Caco-2 cell lines didn't change their metabolic activity and established its safety for intestinal cells. These results are in agreement with Bhat et al. (2019), who found that *L. rhamnosus* MTCC5897 was safe and non-cytotoxic to Caco-2 cells at different doses (10^6-10^{10} CFU/mL) upon incubation for 24 h. Similar to our findings, Singh et al. (2018) have demonstrated that *L. rhamnosus* GG encapsulated with novel cellulose/chitosan-based particles displayed low toxicity prolife against Caco-2 cell line. Likewise, the probiotic strain of *L. fermentum* MTCC5898 was also reported to be non-cytotoxic to human Caco-2 cells (Bhat et al., 2020). Serum contains potent antimicrobial proteins and peptides, such as lactoferrin, bactericidal/permeability increasing proteins, serprocidins, cathelicidins, lysozyme, phospholipases A2, calprotectin, and the complement system (Levy, 2000). The active complement system opsonises the translocated bacteria from the gut to the blood stream and accelerates their phagocytosis by macrophages and leukocytes (Pradhan et al., 2019). When serum is heat-inactivated, all the immune cells and antimicrobial factors get denatured, as these are made up of protein. In the present experiment, the growth of CRD7 and CRD11 in heat-inactivated serum was found to be higher in comparison to active serum. This result indicates that our lactobacilli strains were sensitive to serum-mediated killing, whereas the faecal flora was resistant. Similarly, Vesterlund et al. (2007) found higher survival percentages of lactobacilli strains in human heat-inactivated serum.

LAB produces either the D(−) or L(+) isomer of lactic acid or their combination as one of the main fermentation by-products of carbohydrate metabolism, depending on the environmental conditions. Despite the fact that L-lactic acid is majorly produced during fermentation, a smaller amount of D-lactic acid could also be detected (Fugaban et al., 2021). The human body contains enzymes that are capable of metabolizing only L-lactic acid (Lee et al., 2022). Ingestion of large amounts of

D-lactic acid above the safety threshold, particularly in infants, has been linked to metabolic disorders, intestinal discomfort, and even acidosis, which can lead to sequelae of chronic conditions such as chronic fatigue syndrome (Connolly et al., 2005; Vitetta et al., 2017). In this study, probiotic strains produced trace amounts of D (−)- and high levels of L (+)- lactic acids, which is desirable. In line with our findings, Lee et al. (2022) conducted a similar study with four *Lactobacillus* strains that produced significant amounts of L-lactate, ranging from 14.33 to 28.12 g/L. Consistently, our results obtained for *Enterococcus faecalis* NCDC114 as a reference culture for L-lactic acid production are in agreement with the values reported by Fugaban et al. (2021).

The colonic bacteria can degrade different nitrogenous compounds (e.g., peptides, proteins, and amino acids), which leads to the formation of various toxic by-products (ammonia, phenols, and indoles) that may be hazardous to consumer health (Richardson et al., 2013). An earlier study reported that anaerobic putrefactive bacteria such as *Clostridium perfringens*, *Enterobacter*, *Fusobacterium*, *Peptostreptococcus,* and *Bacillus* spp. are the major producers of ammonia, while lactobacilli can produce trace amounts of ammonia (Smith and Macfarlane, 1996). Therefore, elucidating microbial ammonia production through proteolysis is highly pertinent to human gut health. The present study found no indication of ammonia production by *L. rhamnosus* CRD11 and *L. plantarum* CRD7. This finding may be related to the absence of urease enzyme activity as discussed above, which further supports our obtained data. These results are in agreement with the values and observations reported by Kim et al. (2018).

A correlation matrix was plotted to measure the strength and direction of the relationship between the different variables such as cell cytotoxicity, biogenic amines, resistance to serum, lactic acid production, and mucin degradation activity of *Lactobacillus* strains. The colour scale explains the mean response of probiotic strains towards analyzed data, with yellow and blue indicating the highest to least responses for evaluated parameters. On the other hand, a static heat map depicts the largest and smallest values in the data matrix, as well as clustering of rows and/or rows of similar significance (Tauno, 2016). Although the heatmaps are widely used in visualization of omics studies large data sets, we used it in the present study to analyse the data of *in vitro* safety attributes. As an outcome, four probiotic strains were grouped into two major clusters.

5. Conclusions

A comprehensive safety and toxicity assessment of electrohydrodynamically encapsulated *L. rhamnosus* CRD11 and *L. plantarum* CRD7 showed negative results for the production of biogenic amines, gelatinase, hemolysin, DNase, and mucin degradation activities. Moreover, both strains were sensitive to key therapeutic antibiotics according to EFSA guidelines and did not have genes associated with potential virulence factors. In this modern functional biotics arena, they may serve as good biotherapeutic adjuncts and alternatives to antibiotics. This is further supported by a low toxicity profile for Caco2 cell lines and their susceptibility to serum. Overall, this study provides a way to assess the potential risks of *L. plantarum* CRD7 and *L. rhamnosus* CRD11. CRD7 and CRD11 are found to be safe, non-toxic to human epithelial cells, and excellent probiotic candidates for various food/feed applications. However, future studies are warranted to confirm these *in vitro* safety findings in animal models.

CRediT authorship contribution statement

Vinay Venkatesh Varada: Methodology, Conceptualization, Data curation, Validation, Formal analysis, Writing – original draft. **Divya Panneerselvam:** Methodology, Data curation. **Heartwin A. Pushpadass:** Conceptualization, Project administration, Funding acquisition. **Rashmi Hogarehalli Mallapa:** Writing – review & editing, Supervision. **Chand Ram:** Writing – review & editing. **Sachin Kumar:** Conceptualization, Project administration, Supervision, Funding acquisition, Writing – review & editing, All authors read and approved the manuscript.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

Acknowledgements

The authors are highly thankful to the Director, National Dairy Research Institute, Karnal, India, for the facilities provided. This study was supported by Department of Science & Technology (DST), New Delhi, India (F.No: DST/NM/TAP-AGRI/02/2019(G) NDRI, KARNAL).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100507.

Abbreviation

(MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
(ANOVA) Analysis of Variance
(ARGs) Antibiotic Resistance Genes
(AMR) Antimicrobial resistance
(BA) Biogenic Amine
(BHI) Brain Heart Infusion
(CFS) Cell-Free Supernatant
(CLSI) Clinical and Laboratory Standardization
(EU) European Commission
(MRS) de Mann Rogosa and Sharpe broth
(DMEM) Dulbecco's Modified Eagle's Medium
(EFSA) European Food Safety Authority
(GIT) Gastrointestinal tract
(GRAS) Generally recognized as safe
(HPLC) High-Performance Liquid Chromatography
(LAB) Lactic acid bacteria
(MICs) Minimum Inhibitory Concentrations
(NR) Neutral Red
(OD) Optical Density
(FEEDAP) Panel on Additives and Products or Substances used in Animal Feed
(PAS) Periodic Acid-Schiff
(PCR) Polymerase Chain Reaction
(QPS) Qualified Presumption of Safety
(SEM) Scanning Electron Microscopy
(SDS-PAGE) Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis

References

Abe, F., Muto, M., Yaeshima, T., Iwatsuki, K., Aihara, H., Ohashi, Y., Fujisawa, T., 2010. Safety evaluation of probiotic Bifidobacteria by analysis of mucin degradation activity and translocation ability. Anaerobe 16 (2), 131–136. https://doi.org/10.1016/j.anaerobe.2009.07.006.

Alayande, K.A., Aiyegoro, O.A., Ateba, C.N., 2020. Distribution of important probiotic genes and identification of the biogenic amines produced by *Lactobacillus acidophilus* PNW3. Foods 9 (12), 1840. https://doi.org/10.3390/foods9121840.

Balamurugan, R., Chandragunasekaran, A.S., Chellappan, G., Rajaram, K., Ramamoorthi, G., Ramakrishna, B.S., 2014. Probiotic potential of lactic acid bacteria present in homemade curd in southern India. Indian J. Med. Res. 140, 345.

Bernardeau, M., Vernous, J.P., Henri-Dubernet, S., Gueguen, M., 2008. Safety assessment of dairy microorganisms: the *Lactobacillus* genus. Int. J. Food Microbiol. 126, 278–285. https://doi.org/10.1016/j.ijfoodmicro.2007.08.015.

Bhagwat, A., Annapure, U.S., 2019. *In vitro* assessment of metabolic profile of *Enterococcus* strains of human origin. J. Genet. Eng. Biotechnol 17 (1), 1–11. https://doi.org/10.1186/s43141-019-0009-0.

Bhat, M.I., Kapila, S., Kapila, R., 2020. *Lactobacillus fermentum* (MTCC-5898) supplementation renders prophylactic action against *Escherichia coli* impaired intestinal barrier function through tight junction modulation. LWT (Lebensm.-Wiss. & Technol.) 123, 109118. https://doi.org/10.1016/j.lwt.2020.109118.

Bhat, M.I., Singh, V.K., Sharma, D., Kapila, S., Kapila, R., 2019. Adherence capability and safety assessment of an indigenous probiotic strain *Lactobacillus rhamnosus* MTCC-5897. Microb. Pathog. 130, 120–130. https://doi.org/10.1016/j.micpath.2019.03.009.

Bover-Cid, S., Holzapfel, W.H., 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J. Food Microbiol. 53, 33–41. https://doi.org/10.1016/S0168-1605 (99)00152-X.

Campedelli, I., Mathur, H., Salvetti, E., Clarke, S., Rea, M.C., Torriani, S., O'Toole, P.W., 2019. Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. Appl. Environ. Microbiol. 85 (1), e01738 https://doi.org/10.1128/AEM.01738-18, 18.

Carlet, J., 2012. The gut is the epicentre of antibiotic resistance. Antimicrob. Resist. Infect. Control 1, 1–7. https://doi.org/10.1186/2047-2994-1-39.

Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8 (2), 130–132. https://doi.org/10.1093/clinchem/8.2.130.

Charteris, C., Kelly, K., Morelli, M., Collins, C., 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84 (5), 759–768. https://doi.org/10.1046/j.1365-2672.1998.00407.x.

Clinical and Laboratory Standards Institute (CLSI), 2015. Performance Standards for Antimicrobial Susceptibility Testing: 25th Informational Supplement. Clinical and Laboratory Standards Institute, Pittsburgh, PA. CLSI Document M100-S25.

Coelho-Rocha, N.D., De Castro, C.P., De Jesus, L.C., Leclercq, S.Y., de CiccoSandes, S.H., Nunes, A.C., Mancha-Agresti, P., 2018. Microencapsulation of lactic acid bacteria improves the gastrointestinal delivery and in situ expression of recombinant fluorescent protein. Front. Microbiol. 9, 2398. https://doi.org/10.3389/fmicb.2018.02398.

Connolly, E., Abrahamsson, T., Bjorksten, B., 2005. Safety of D (-)-lactic acid producing bacteria in the human infant. J. Pediatr. Gastroenterol. Nutr. 41 (4), 489–492. https://doi.org/10.1097/01.mpg.0000176179.81638.45.

Das, D.J., Shankar, A., Johnson, J.B., Thomas, S., 2020b. Critical insights into antibiotic resistance transferability in probiotic *Lactobacillus*. Nutrition 69, 110567. https://doi.org/10.1016/j.nut.2019.110567.

Das, S., Mishra, B.K., Hati, S., 2020a. Techno-functional characterization of indigenous *Lactobacillus* isolates from the traditional fermented foods of Meghalaya, India. Curr. Res. Food Sci. 3, 9–18. https://doi.org/10.1016/j.crfs.2020.01.002.

De Groote, M.A., Frank, D.N., Dowell, E., Glode, M.P., Pace, N.R., 2005. *Lactobacillus rhamnosus* GG bacteraemia associated with probiotic use in a child with short gut syndrome. Pediatr. Infect. Dis. J. 24, 278–280. https://doi.org/10.1097/01.inf.0000154588.79356.e6.

Dodoo, C.C., Wang, J., Basit, A.W., Stapleton, P., Gaisford, S., 2017. Targeted delivery of probiotics to enhance gastrointestinal stability and intestinal colonisation. Int. J. Pharm. 530 (1–2), 224–229. https://doi.org/10.1016/j.ijpharm.2017.07.068.

Duman, D., Karadag, A., 2021. Inulin added electrospun composite nanofibres by electrospinning for the encapsulation of probiotics: characterisation and assessment of viability during storage and simulated gastrointestinal digestion. Int. J. Food Sci. Technol. 56 (2), 927–935. https://doi.org/10.1111/ijfs.14744.

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B.J., Boobis, A., Kleiner, J., 2002. Methods of in vitro toxicology. Food Chem. Toxicol. 40 (2–3), 193–236. https://doi.org/10.1016/S0278-6915(01)00118-1.

European Food Safety Authority (EFSA), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 5 (12), 587. https://doi.org/10.2903/j.efsa.2007.587.

European Food Safety Authority (EFSA), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10, 2740. https://doi.org/10.2903/j.efsa.2012.2740.

FAO/WHO, 2006. Probiotics in Food. Health and Nutritional Properties and Guidelines for Evaluation. Rome: FAO Food and Nutrition Paper. http://www.fao.org/3/a-a0512e.pdf.

Fouhy, F., Guinane, C.M., Hussey, S., Wall, R., Ryan, C.A., Dempsey, E.M., Cotter, P.D., 2012. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob. Agents Chemother. 56 (11), 5811–5820. https://doi.org/10.1128/AAC.00789-12.

Frenkel, E.S., Ribbeck, K., 2015. Salivary mucins protect surfaces from colonization by cariogenic bacteria. Appl. Environ. Microbiol. 81, 332–338. https://doi.org/10.1128/AEM.02573- 14.

Fugaban, J.I.I., Holzapfel, W.H., Todorov, S.D., 2021. Probiotic potential and safety assessment of bacteriocinogenic *Enterococcus faecium* strains with antibacterial activity against *Listeria* and vancomycin-resistant enterococci. Curr. Res. Microb. Sci. 2, 100070 https://doi.org/10.1016/j.crmicr.2021.100070.

Ganguly, N.K., Bhattacharya, S.K., Sesikeran, B., Nair, G.B., Ramakrishna, B.S., Sachdev, H.P.S., Batish, V.K., Kanagasabapathy, A.S., Muthuswamy, V., Kathuria, S. C., 2011. ICMR-DBT guidelines for evaluation of probiotics in food. Indian J. Med. Res. 134, 22.

Gharbi, Y., Fhoula, I., Ruas-Madiedo, P., Afef, N., Boudabous, A., Gueimonde, M., Ouzari, H.I., 2019. In-vitro characterization of potentially probiotic *Lactobacillus* strains isolated from human microbiota: interaction with pathogenic bacteria and the enteric cell line HT29. Ann. Microbiol. 69 (1), 61–72. https://doi.org/10.1007/s13213-018-1396-1.

Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L.V., Urdaci, M.C., Cutting, S.M., 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. J. Appl. Microbiol. 105 (2), 510–520. https://doi.org/10.1111/j.1365-2672.2008.03773.x.

Hussein, W.E., Abdelhamid, A.G., Rocha-Mendoza, D., García-Cano, I., Yousef, A.E., 2020. Assessment of safety and probiotic traits of *Enterococcus durans* OSY-EGY, isolated from Egyptian artisanal cheese, using comparative genomics and phenotypic analyses. Front. Microbiol. 11, 608314 https://doi.org/10.3389/fmicb.2020.608314.

Huys, G., Botteldoorn, N., Delvigne, F., De Vuyst, L., Heyndrickx, M., Pot, B., Daube, G., 2013. Microbial characterization of probiotics–advisory report of the working group "8651 probiotics" of the Belgian superior health council (SHC). Mol. Nutr. Food Res. 57 (8), 1479–1504. https://doi.org/10.1002/mnfr.201300065.

Jansen, S.C., van Dusseldorp, M., Bottema, K.C., Dubois, A.E., 2003. Intolerance to dietary biogenic amines: a review. Ann. Allergy Asthma Immunol. 91 (3), 233–241. https://doi.org/10.1016/S1081-1206 (10)63523-5.

Kim, M.J., Ku, S., Kim, S.Y., Lee, H.H., Jin, H., Kang, S., Ji, G.E., 2018. Safety evaluations of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI. Int. J. Mol. Sci. 19 (5), 1422. https://doi.org/10.3390/ijms19051422.

Koyama, S., Fujita, H., Shimosato, T., Kamijo, A., Ishiyama, Y., Yamamoto, E., 2018. Septicemia from *Lactobacillus rhamnosus* GG, from a probiotic enriched yogurt, in a patient with autologous stem cell transplantation. Probiotics Antimicrob. Proteins 11 (1), 295–298. https://doi.org/10.1007/s12602-018-9399-6.

Kumar, S., Pattanaik, A.K., Sharma, S., Jadhav, S.E., Dutta, N., Kumar, A., 2017b. Probiotic potential of a *Lactobacillus* bacterium of canine faecal-origin and its impact on select gut health indices and immune response of dogs. Probiotics Antimicrob. Proteins 9, 262–277. https://doi.org/10.1007/s12602-017-9256-z.

Kumar, S., Tariq, H., Singh, A., Tyagi, N., Mondal, G., Gupta, M., Tyagi, A.K., 2017a. Significance of probiotics as feed additives in livestock and poultry nutrition. Indian J. Anim. Nutr. 34 (4), 361–373.

Kumar, S., Pattanaik, A.K., Jadhav, S.E., 2021. Potent health-promoting effects of a synbiotic formulation prepared from *Lactobacillus acidophilus* NCDC15 fermented milk and *Cichorium intybus* root powder in Labrador dogs. Curr Res Biotechnol 1 (3), 209–214.

Kumar, S., Varada, V.V., Banakar, P.S., Tyagi, N., Chouraddi, R., Hogarehalli Mallapa, R., Tyagi, A.K., 2022. Screening and characterization of Sahiwal cattle calves-origin lactic acid bacteria based on desired probiotic attributes for potential application. Anim. Biotechnol. 1–14 https://doi.org/10.1080/10495398.2022.2043885.

Lee, Y.R., Bang, W.Y., Baek, K.R., Kim, G.H., Kang, M.J., Yang, J., Seo, S.O., 2022. Safety evaluation by phenotypic and genomic characterization of four lactobacilli strains with probiotic properties. Microorganisms 2218. https://doi.org/10.3390/microorganisms10112218.

Levy, O., 2000. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood 96 (8), 2664–2672. https://doi.org/10.1182/blood.V96.8.2664.

Liong, M.T., 2008. Safety of probiotics: translocation and infection. Nutr. Rev. 66 (4), 192–202. https://doi.org/10.1111/j.1753-4887.2008.00024.x.

Liu, S., Xiao, H., Xiong, Y., Chen, J., Wu, Q., Wen, X., Wang, L., 2022. Effects of fermented feed on the growth performance, intestinal function, and microbiota of piglets weaned at different age. Front. Vet. Sci. 9 https://doi.org/10.3389/fvets.2022.841762.

Lu, H., Zhao, W., Liu, W.H., Sun, T., Lou, H., Wei, T., Chen, Q., 2021. Safety evaluation of *Bifidobacterium lactis* BL-99 and *Lacticaseibacillus paracasei* K56 and ET-22 in vitro and in vivo. Front. Microbiol. 12, 686541. https://doi:10.3389/fmicb.2021.686541.

Ma, J., Xu, C., Yu, H., Feng, Z., Yu, W., Gu, L., Hou, J., 2021. Electro-encapsulation of probiotics in gum Arabic-pullulan blend nanofibres using electrospinning technology. Food Hydrocolloids 111, 106381. https://doi.org/10.1016/j.foodhyd.2020.106381.

Markowiak, P., Slizewska, K., 2017. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients 9 (9), 1021. https://doi: 10.3390/nu9091021.

Moayyedi, M., Eskandari, M.H., Rad, A.H.E., Ziaee, E., Khodaparast, M.H.H., Golmakani, M.T., 2018. Effect of drying methods (electrospraying, freeze drying and spray drying) on survival and viability of microencapsulated *Lactobacillus rhamnosus* ATCC 7469. J. Funct.Foods 40, 391–399. https://doi.org/10.1016/j.jff.2017.11.016.

Parsana, Y., Yadav, M., Kumar, S., 2023. Microencapsulation in the chitosan-coated alginate-inulin matrix of *Limosilactobacillus reuteri* SW23 and *Lactobacillus salivarius* RBL50 and their characterization. Carbohydr. Polym. Technol.Appli., 100285 https://doi.org/10.1016/j.carpta.2023.100285.

Pattanaik, A.K., Kumar, S., Rashmi, H.M., 2022. Probiotics, prebiotics, eubiotics and synbiotics for human and animal health and mitigation of antimicrobial resistance. Indian J. Comp. Microbiol. Immunol. Infect. Dis. 43, 90–97.

Pradhan, D., Mallappa, R.H., Grover, S., 2020. Comprehensive approaches for assessing the safety of probiotic bacteria. Food Control 108, 106872. https://doi: 10.1016/j.foodcont.2019.106872.

Pradhan, D., Singh, R., Tyagi, A., Rashmi, H.M., Batish, V.K., Grover, S., 2019. Assessing safety of *Lactobacillus plantarum* MTCC 5690 and *Lactobacillus fermentum* MTCC 5689 using *in vitro* approaches and an *in vivo* murine model. Regul. Toxicol. Pharmacol. 101, 1–11. https://doi.org/10.1016/j.yrtph.2018.10.011.

Priyadarshani, W.M.D., Rakshit, S.K., 2011. Screening selected strains of probiotic lactic acid bacteria for their ability to produce biogenic amines (histamine and tyramine). Int. J. Food Sci. Technol. 46 (10), 2062–2069. https://doi.org/10.1111/j.1365-2621.2011.02717.x.

Rastogi, S., Mittal, V., Singh, A., 2020. *In vitro* evaluation of probiotic potential and safety assessment of *Lactobacillus mucosae* strains isolated from Donkey's lactation. Probiotics Antimicrob. Proteins 12, 1045–1056. https://doi.org/10.1007/s12602-019-09610-0.

Reuben, R.C., Roy, P.C., Sarkar, S.L., Alam, R.U., Jahid, I.K., 2019. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. BMC Microbiol. 19, 253. https://doi.org/10.1186/s12866-019-1626-0.

Richardson, A.J., McKain, N., Wallace, R.J., 2013. Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids. BMC Microbiol. 13 (1), 1–8. https://doi.org/10.1186/1471-2180-13-6.

Russo, M., Marquez, A., Herrera, H., Abeijon-Mukdsi, C., Saavedra, L., Hebert, E., Gauffin- Cano, P., Medina, R., 2020. Oral administration of *Lactobacillus fermentum* CRL1446 improves biomarkers of metabolic syndrome in mice fed a high-fat diet supplemented with wheat bran. Food Funct. 11 (5), 3879–3894. https://doi.org/10.1039/D0FO00730G.

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M., de, L., Bories, G., Chesson, A., Cocconcelli, P.S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., Lopez-Alonso, M., LopezPuente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., de Villa, R., Wallace, R., Wester, P., Anguita, M., Galobart, J., Innocenti, M., Martino, L., 2017. EFSA panel on additives and products or substances used in animal feed (FEEDAP). Guid. Assess. Saf. Feed Additiv. Target Species. EFSA J. 15 (10) https://doi.org/10.2903/j.efsa.2017.5021.

Saqib, S., Akram, A., Halim, S.A., Tassaduq, R., 2017. Sources of β-galactosidase and its applications in food industry. 3 Biotech 7 (1). https://doi.org/10.1007/s13205-017-0645-5.

Singh, A., Kumar, S., Vinay, V.V., Tyagi, B., Choudhury, P.K., Rashmi, H.M., Banakar, P. S., Tyagi, N., Tyagi, A.K., 2021b. Autochthonous *Lactobacillus* spp. isolated from Murrah buffalo calves show potential application as probiotic. Curr. Res. Biotechnol. 3, 109–119. https://doi.org/10.1016/j.crbiot.2021.04.002.

Singh, M., Kumar, S., Banakar, P.S., Vinay, V.V., Das, A., Tyagi, N., Tyagi, A.K., 2021a. Synbiotic formulation of *Cichorium intybus* root powder with *Lactobacillus acidophilus* NCDC15 and *Lactobacillus reuteri* BFE7 improves growth performance in Murrah buffalo calves via altering selective gut health indices. Trop. Anim. Health Prod. 53 (2), 1–9. https://doi.org/10.1007/s11250-021-02733-z.

Singh, P., Medronho, B., dos Santos, T., Nunes-Correia, I., Granja, P., Miguel, M.G., Lindman, B., 2018. On the viability, cytotoxicity and stability of probiotic bacteria entrapped in cellulose-based particles. Food Hydrocolloids 82, 457–465. https://doi.org/10.1016/j.foodhyd.2018.04.027.

Smith, E.A., Macfarlane, G.T., 1996. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. J. Appl. Bacteriol. 81 (3), 288–302. https://doi.org/10.1111/j.1365-2672.1996.tb04331.x.

Su, G.L., W, C., Ko, P., Bercik, Y., Falck-Ytter, S., Sultan, A.V., WeizmanMorgan, R.L., 2020. AGA clinical practice guidelines on the role of probiotics in the management of gastrointestinal disorders. Gastroenterology 159 (2), 697–705. https://doi.org/10.1053/j.gastro.2020.05.059.

Thumu, S.C.R., Halami, P.M., 2020. In vivo safety assessment of *Lactobacillus fermentum* strains, evaluation of their cholesterol-lowering ability and intestinal microbial modulation. J. Sci. Food Agric. 100 (2), 705–713. https://doi.org/10.1002/jsfa.10071.

Tauno, M., 2016. Statistical analysis of multivariate data in bioinformatics. Published online. http://dspace.ut.ee/bitstream/handle/10062/50409/metsalu_tauno.pdf.

Umar, H., Choo, S., Desa, M.N.M., Rangasamy, P., Than, L.T.L., 2020. Isolation and safety characterisation of *Lactobacilli* strains with antimicrobial properties as potential probiotics for human use. LWT (Lebensm.-Wiss. & Technol.) 131, 109796. https://doi.org/10.1016/j.lwt.2020.109796.

Varada, V.V., Kumar, S., Chhotaray, S., Tyagi, A.K., 2022b. Host-specific probiotics feeding influence growth, gut microbiota, and fecal biomarkers in buffalo calves. Amb. Express 12 (1), 1–18. https://doi.org/10.1186/s13568-022-01460-4.

Varada, V.V., Tyagi, A.K., Banakar, P.S., Das, A., Tyagi, N., Mallapa, R.H., Kumar, S., 2022a. Autochthonous *Limosilactobacillus reuteri* BFE7 and *Ligilactobacillus salivarius* BF17 probiotics consortium supplementation improves performance, immunity, and selected gut health indices in Murrah buffalo calves. Vet. Res. Commun. 46, 757–767. https://doi:10.1007/s11259-022-09896-6.

Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., Ouwehand, A.C., 2007. Safety assessment of *Lactobacillus* strains: presence of putative risk factors in faecal, blood and probiotic isolates. Int. J. Food Microbiol. 116 (3), 325–331. https://doi.org/10.1016/j.ijfoodmicro.2007.02.002.

Vitetta, L., Coulson, S., Thomsen, M., Nguyen, T., Hall, S., 2017. Probiotics, D–Lactic acidosis, oxidative stress and strain specificity. Gut Microb. 8 (4), 311–322. https://doi.org/10.1080/19490976.2017.1279379.

Yakabe, T., Moore, E.L., Yokota, S., Sui, H., Nobuta, Y., Fukao, M., Yajima, N., 2009. Safety assessment of *Lactobacillus brevis* KB290 as a probiotic strain. Food Chem. Toxicol. 47 (10), 2450–2453. https://doi.org/10.1016/j.fct.2009.07.001.

Zhou, J.S., Gopal, P.K., Gill, H.S., 2001. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. Int. J. Food Microbiol. 63, 81–90. https://doi.org/10.1016/S0168-1605(00)00398-6.