Probiotic properties of *Lactobacillus casei* – MYSRD 108 and *Lactobacillus plantarum*-MYSRD 71 with potential antimicrobial activity against *Salmonella paratyphi*

S. Divyashree , P.G. Anjali , Rakesh Somashekaraiah , M.Y. Sreenivasa [*]

Department of Studies in Microbiology, University of Mysore, Mysuru, Karnataka, India

ABSTRACT

A total of 130 isolates were screened, twelve isolates were characterized for probiotic attributes and two isolates with best probiotic features were evaluated in the study. Isolates MYSRD108 and MYSRD71 survived gastric conditions and were susceptible to tested antibiotics. Isolates showed more vital cell surface traits such as autoaggregation of 89.2 and 88.5% and cell surface hydrophobicity of 61 and 64%. PCR amplification followed by 16sRNA sequencing results confirmed that the isolates as *Lactobacillus casei* (MYSRD 108) and *Lactobacillus plantarum* (MYSRD 71). During this study, the Cells and their Cell Free Supernatant (CFS) were examined for antimicrobial activity. Both the isolates inhibited different bacterial pathogens in which the growth of *S. paratyphi* was significantly reduced. Further, their CFS also showed inhibitory effects against *S. paratyphi* with agar well diffusion and Minimum Inhibitory Concentration using Broth micro dilution method. The antimicrobial compounds in the CFS was characterized to different constraints such as pH neutralization, heat treatment, Hydrogen peroxide test and storage stability at -20> °C and represented that the antagonistic acitivity against *Salmonella* is due to the presence of organic acids in the supernatants that lowered the pH. These strains were further examined for the inhibition of *S. paratyphi* biofilm. The results indicated that CFS reduced *S. paratyphi* biofilm by more than 75% and the number of *Salmonella* biofilm was effectively reduced using 15% concentration of CFS. These strains may be used to produce antimicrobial compounds which can be a substitute for chemical preservatives in food industry.

1. Introduction

Food-borne illnesses are a growing worldwide awareness as they are encouraged for increased morbidity and mortality. About 600 million cases of food-borne infections with 31 global food-borne hazards caused more than 400,000 deaths (WHO 2015). The leading cause of food-borne diseases due to pathogenic microorganisms that may be transmitted through contaminated foods. The contamination of food and its products due to food-borne pathogens results in biofilms which are a significant threat factor. The food-borne disease outbreaks in France due to biofilms contamination in equipment contributing to 59% [1]. The important bacteria which are known to cause food-borne diseases includes *Staphylococcus spp, Campylobacter jejuni, Clostridium spp, Escherichia coli, Brucella, Listeria* spp, *Salmonella and Shigella* spp., *Vibrio* spp etc.

Along with bacteria, moulds and their mycotoxins are common spoilage organisms in various food and feed products. These spoiling moulds cause significant economic losses worldwide. The primary species involved in the food spoilage are *Aspergillus* spp, *Fusarium spp, Penicillium* sppand *Mucor* spp *etc.* [2].

Among food-borne microorganisms, *Salmonella* is one of the major causes of food-borne illnesses in humans, in which *Salmonella typhi* and *Salmonella paratyphi* are most common to cause diseases in human's results in typhoid and paratyphoid fever, respectively. The major virulence factor in salmonella is their biofilm production adds to the *Salmonella* persistence in the human and animal gut resulting in primary infection such as septicemia, leukopenia, immunological and neurological symptoms [3]. The Salmonella biofilms also encourage virulence factors, antimicrobial resistance, and mechanical persistence, increasing the microorganisms' survival [4].

Salmonella species can colonize and conquer the small intestine and colon mucosa by frequently adhering to the gastrointestinal tract. The

antimicrobial substances produced by the intestinal microorganisms play a significant role in fighting against *Salmonella*. For some decades, treatment with antibiotics has been successfully used as therapeutic and prophylactic agents to obviate colonization, as well as invasive infections [5]. However, the efficacy is compromised due to an overuse of antibiotics that may alter the intestinal microflora composition and increase antibiotic-resistant and make the patient more susceptible to *Salmonella* infection. The antibiotic-resistant salmonella can prolong the carriage time and significantly decreases the effectiveness of existing treatment approaches, affecting patient to spread and shed the salmonella bacteria in feces for a long time frame, increasing the persistence of the illness [6]. Hence, alternative therapies have been explored for microorganism's infections, including vaccines' development [7], using natural products such as peptides, oil and phytochemicals. Although promising, these compounds' toxicities and their bio-tolerance are of concern, and they are yet in the experimental stages of development [8].

Due to these concerns, the use of probiotic bacteria has been proposed as an alternative prophylactic and therapeutic mode of treatment against *S. paratyphi*. A very promising approach for preventing biofilm formation in the food industry and antimicrobial resistance is probiotics to colonize the hard surfaces to prevent the colonization of bacterial pathogen species, using the competitive exclusion principle [9]. The application of probiotics is to treat and prevent a wide variety of illnesses has gained favor to find alternatives to traditional therapies such as antibiotics, gastrointestinal and other diseases because of the lack of suitable treatments in the past decade. Consumption of probiotics is associated with health benefits, including stimulation of the immune system, exclusion and antagonism for defense against diarrheal diseases, nosocomial and respiratory tract infections, reduction in serum cholesterol, attenuation of overt immune-inflammatory disorders and anticancer effects [10]. Lactic acid bacteria (LAB) are the most common probiotic have traditionally been used as natural bio preservatives in food and animal feed and food-borne pathogens by their antimicrobial compounds such as organic acids, bacteriocin and hydrogen peroxide [11]. LAB have received much attention, primarily because of their food-grade and GRAS status [12,13].

This study's objectives were to characterize LAB isolated from fermented foods, evaluated their probiotic attributes like gastric juice and bile tolerances, antibiotic susceptibility profile, cell surface hydrophobicity, auto-aggregation abilities to assess further the antimicrobial activity of LAB against *S. paratyphi*. The potential isolates' CFS was examined for their ability to inhibit *S. paratyphi* and their biofilm production.

2. Materials and methods

2.1. Bacterial isolates, identification and characterization of LAB

The LAB isolates were obtained and screened from the Lactic Acid Bacteria (LAB) library having approximately 130 isolates isolated from different fermented foods and maintained at Mycotoxicology laboratory, Department of studies in Microbiology, University of Mysore. Essential characterization of the LAB isolates was performed through grams reaction, morphology, catalase test motility, bile salt hydrolase, carbohydrate fermentation test with different carbohydrate viz arabinose, sorbitol, maltose, sucrose, mannitol and lactose (Hi –media). The LAB isolates were further characterized for growth at different temperatures and pH. The potential probiotic isolates MYSRD 108 and MYSRD 71 was selected for further characterization and presented in this study, which was isolated from fermented food Vellappam.

2.1.1. Screening of LAB for antibacterial and antifungal activity

The Antibacterial activity of LAB isolates was tested against some of the most common pathogens using microplates assay described by Jamwal et al. [14]. With slight modifications. Pathogens used as indicators strains. *E. coli* (ATCC 25,922), *Klebsiella pneumoniae* (MTCC

7407), *Pseudomonas aeruginosa* (ATCC 15,422), *Staphylococcus aureus* (ATCC 6538) and *Salmonella paratyphi* (ATCC 9150) obtained from American type culture collection (ATCC) and Microbial type culture collection (MTCC). The overnight grown LAB isolates at 37 °C for 24 h were centrifuged at 8000 rpm for 10 mins at 4 °C, and the spent medium was collected in fresh tubes. The spent culture medium was filtered through 25 mm/0.45 μm nylon membrane filters (Millipore) and stored at 20 °C for further use. The cell-free supernatant (of 10, 15 and 20% concentration) and 50 μL of bacterial suspension containing 10^8 CFU/mL were filled in a sterile 96-well microtiter plate. Then made up 200 μL using Luria-Bertani (LB) broth. The LB broth containing bacterial suspension was considered the positive control, and the un-inoculated LB broth was considered as negative control. The O.D at 600 nm was measured after incubating 96 well plates at 37 °C for 24 h. The total inhibition percentage of the pathogen was calculated using the equation: [(OD of positive control – OD of the test sample)/OD of positive control] *100.

Eight fungal species, *Fusarium sporotrichiodes, F. equiseti, F. poea, F. avenaceum, F. graminearum, F. verticillioides, Aspergillus parasiticus* and *Rhizopus oryzae,* were chosen to determine the antifungal activities of LAB isolates using the agar overlay method described by Magnusson and Schnurer [15] with slight modifications. The overnight LAB cultured were inoculated on MRS agar plates in 2 cm wide lines and incubated at 37 °C for 24 h. After incubation, the MRS plates were overlaid with PDA soft agar containing 20 μL fungal spore suspension ($\sim10^6$ spores/mL). The plates were incubated at 25 °C for 7 days. The leaves were examined for the apparent inhibition zone around the streaked area of LAB isolates and measured.

2.1.2. Species identification of LAB

Identification of LAB was made primarily by partial sequencing of 16S rRNA genes. Genomic DNA of the potential probiotic isolates was extracted using the phenol-chloroform method. PCR was performed using the forward primer 8F-5′AGAGTTTGATCCTGGCTCAG 3′[16] and reverse primer 1391R-5′GACGGGCGGTGWGTRCA 3′[17]. The PCR products are further verified by 1% agarose gel electrophoresis and subjected to 16S rRNA gene sequencing. The identification of the LAB isolates was carried out by comparing with reference sequences through the BLAST program. A phylogenetic tree was constructed using the neighbor-joining method with the MEGA 5.1 software programmer. The sequences with the GenBank database using the BLASTN program available on the NCBI server [18].

2.2. Evaluation of probiotic attributes

2.2.1. Bile tolerance and acidic pH tolerance

The isolates' ability to survive in the presence of acidic pH and bile salt was investigated as per Deepthi et al. [19] with slight modification. Overnight bacterial isolates were grown in MRS broth at 37 °C, sub-cultured to fresh MRS broth, adjusted to different pH values 2,4 and 6.5 using 5 M HCL and incubated at 37 °C for 0–5 h. After incubation, the plating was done and incubated anaerobically for 24–48 h at 37 °C. The tolerance of LAB isolates to acidic pH was determined by evaluating the CFU/ mL in all the test MRS agar plates by comparing with control plates. LAB isolates were subcultured to fresh MRS broth supplemented with 0.3% oxgall, and without oxgall was considered as control for resistance to bile salts. The samples are incubated at 37 °C for time intervals 0–5 h. After incubation, aliquots of LAB isolates are plated on MRS agar plates and incubated at 37 °C for 24–48 h. The LAB viable count was compared using the equations (%) = biomass at time (t) / biomass at initial time (o)*100.

2.2.2. Antibiotic susceptibility test

The susceptibility to antibiotics of the LAB isolates to nine commonly used antibiotics was assessed by a disk diffusion method (Kirby-Bauer method) for the following antibiotics proposed in accordance with

European food safety authority guidelines: clindamycin (2mcg), streptomycin (10mcg), vancomycin (30mcg), tetracycline (30mcg), chloramphenicol (30mcg), erythromycin (15mcg), tylosin (15mcg), kanamycin (30mcg), and gentamycin (10mcg). The MRS agar plate was overlaid with overnight 100 μL of LAB inoculums containing 10^8 CFU/mL and antibiotics discs under sterile conditions. Then MRS plates were incubated at 37 °C for 16–24 h under anaerobic conditions. The susceptibility to antimicrobials of LAB isolates was assessed by measuring the diameter of the inhibition zone of bacterial growth using the antibiotic zone scale, and results interpreted as resistance, intermediate and susceptibility following CLSI guidelines.

2.2.3. Auto aggregation

Auto aggregation abilities of LAB isolates were measured as per the method described by Angmo et al. [20] with slight modification. The overnight grown LAB culture in MRS broth was centrifuged at 8000 rpm for 10 min at 4 °C and washed twice with 1 mL PBS. Cells were resuspended in 5 mL of PBS buffer and mixed by vortexing for 10 s to give 10^8 CFU/mL by adjusting to 0.25 ± 0.05 OD at 600 nm, then incubated for 5 h at room temperature. The OD is taken at each hour internal (0–5 h) by taking only the upper suspension carefully, transferred to microtitre plates, and an auto-aggregation percentage was measured at 620 nm absorbance using the formula: $1-(A_t/A_o)*100$. A_t represents the absorbance at a particular time $t = 1, 2, 3, 4$ and 5 h and A_o the absorbance at time $t = 0$.

2.2.4. Cell surface hydrophobicity

The degree of cell surface hydrophobicity of the LAB isolates was assessed by microbial adhesion to hydrocarbons was determined by xylene extraction according to the method of the Lee et al. [21] with slight modifications. The bacterial or LAB suspension was prepared as described as an autoaggregation assay (A600 nm). The cell suspension of 3 mL was mixed with an equal volume of xylene and further vortexing for 3 min to mix the two-phase system. After one hour of incubation at room temperature, the upper aqueous layer was removed, and OD was measured at 600 nm. Similarly, OD is taken for 2, 3, 4 and 5 h time intervals. Hydrophobicity was evaluated as the percentage decrease in the OD of a bacterial suspension at the initial aqueous layer and calculated using the equation: $(A_o-A/A_o)*100$. A_o represents the absorbance before extraction with an organic solvent, and A represents the absorbance after extraction with an organic solvent.

2.3. Evaluation of anti-salmonella attributes

2.3.1. Evaluation of the antimicrobial activity of selected LAB isolates using agar well diffusion

To measure antimicrobial activity against common food pathogenic bacteria, *S. paratyphi was* obtained from ATCC 9150, and Lactobacillus cell-free supernatant was obtained as described in Section 2.1.1. Agar well diffusion method was performed according to Mohanty and Ray [22] with slight modification. The indicator strain *S. paratyphi* about 50 μL/200 mL were added to the Mueller-Hinton agar (MHA) and poured on the Petri plates, after which 6 mm diameter wells were prepared, and cell-free supernatants (from 25 to 325 μL/well) of the LAB isolates were loaded in the wells marked with the isolates names properly. After 24 h incubation at 37 °C, the diameter of inhibition zones was recorded and expressed as (mean ± standards deviation).

2.3.2. Determination of MIC using 96-well microliter plate

The broth microdilution method was used to evaluate the minimum inhibitory concentration (MIC) using a 96-well plate. The overnight culture of active *S. paratyphi* (20 μL) was added into a 96-well plate, and the CFS was used at different concentrations (i.e. 2, 4, 8, 12, 15, 25, 35, 45 and 50%) and made the final 200 μL volume using Luria-Bertani (LB) broth. The pathogen inoculated LB broth is considered as the positive control, and uninoculated LB broth is considered as a negative control.

Absorbance of each well was measured by incubating at 37 °C for 0–24 h at 600 nm.

2.3.3. In-vitro time-kill assay

The cell-free supernatant of LAB isolates activity towards *S. paratyphi* was demonstrated by an *in-vitro* killing assay as per the protocol described by Xinlong et al. [23] with slight modification. The overnight cultured LAB isolates were centrifuged, and the cell-free supernatant obtained was syringe filtered. The 300 μL of *S. paratyphi* containing 10^8 CFU/mL in exponential phase were added to 15 mL of CFS, which adjusted to pH 6.5. The MRS broth adjusted to pH 6.5 considered control and incubated at 37 °C for 0, 4, 8, 12, 16, 20 and 24 h followed by serial dilutions and plating on LB agar to determine the viable cells *S. paratyphi*.

2.3.4. Lactobacillus and salmonella co-culture experiment

the assay was demonstrated to evaluate the influence of LAB isolates on the growth of *S. paratyphi* as per the protocol described by Adetoye et al. [24].The assay was performed in 5 mL of double-strength MRS broth and 5 mL of double-strength Mueller Hinton broth followed by MRS-MH broth to assist both the *Lactobacillus* and *Salmonella* growth. For the co-culture experiment, an inoculum of 10^8 CFU/mL of both the organisms was inoculated to MRS-MH broth. The *S. paratyphi* and *Lactobacillus* were cultured alone and considered as control. The experiment samples are incubated for times 0, 8, 16 and 24 h, serially diluted and plated on MRS and MH agar. Similarly, the above procedure was repeated for the co-culture mixture serially diluted at initial and predetermined intervals and plated on MRS agar and MH agar for each organism's growth. The survival cells were compared with the control.

2.3.5. Biofilm assay

The quantification of biofilm formation for potential probiotics and *S. paratyphi* was performed using a microliter plate-based crystal violet staining described by Borges et al. [25] with slight modifications. The overnight cultures of LAB are standardized to 10^7–10^8 CFU/mL by adjusting to 0.25 ± 0.05 OD at 600 nm using PBS buffer, and 20 μL of overnight LAB cultures and *S. paratyphi* was added to each 96-well containing 180 μL of MRS broth and BHI broth respectively. The plates were incubated aerobically for 72 h at 30 °C. Then the non-adherent bacteria were removed by washing wells gently with 200 μL of PBS buffer three times, 100 μL of 0.4% crystal violets stain is added to each well and stained for 30 min. The excess stain is discarded, the dye was gently washed with 200 μL of PBS buffer for 3 times and the dye bound to cells was solubilized with 100 μL of ethanol per well. The uninoculated MRS and BHI broth were considered as a negative control. The optical density was measured at 600 nm using a microplate reader.

2.3.6. Biofilm inhibition assay using crystal violet method and MTT

The biofilm inhibition assay was demonstrated using crystal violet assay as described by Sharma et al. [26] and MTT assay as the protocol described by Wijesundara and Rupasinghe [27] with slight modifications in 96-well polystyrene plate. The *S. paratyphi* suspension of about 20 μL followed by 30 μL of CFS was added to each well and made the final volume to 200 μL using BHI broth. The plates were incubated for 72 h at 37 °C. After incubation, evaluation of biofilm formation was tested as described above. However, for the MTT assay, the non-adherent bacterial cells are removed by PBS washing and the 100 μL freshly prepared BHI broth were added to each well with 10 μL of 12 mM 3-(4,5-dimethylthiazol-2-y1)−2, 5-diphenyltetrazolium bromide (MTT) was then poured to each well then the plates were further incubated for 3 h at 37 °C. The uninoculated pathogen in BHI broth was considered as a negative control, and inoculated pathogen suspension in BHI broth was considered as a positive control. The reduction of MTT due to the activity of living *S. paratyphi* dehydrogenase enzymes results in insoluble purple formazan was quantified at 590 nm using a microtiter reader. The percentage inhibition of biofilm was calculated using the

formula: [(OD of positive control – OD of test samples)/OD of positive control]*100.

2.3.7. SEM analysis

For the microscopic images, the bacterial suspension of *S. paratyphi* was grown in a 12-well tissue culture plate supplemented with sterile glass cover clips followed with 15% of CFS from LAB strains (600 μL/ well) and the wells without CFS was considered as positive control followed by incubation for 72 h at 37 °C. After incubation, the non-adherent cells were removed by washing through PBS buffer or sterile distilled water, and cover clips are air-dried. The bacteria cells were fixed on cover clips in 3% glutaraldehyde for 1 h at 4 °C, and then the samples were washed in 0.1 m phosphate-buffered saline and subsequently incubated in a 2% arginine for 18 h at room temperature. The samples are then subjected to dehydration with ethanol for 15 min and dried for 6 h. Later the coverslips are coated with gold-palladium alloy and observed under Scanning Electron Microscope [28].

2.4. Characterization of the antimicrobial substances

The cell-free supernatant of LAB isolates was evaluated for the production of antimicrobial substances viz organic acids, hydrogen peroxide and bacteriocin using broth microdilution method using a 96-well plate as per Shokryazdan et al. [29] with slight modifications. The overnight cultures of LAB isolates were centrifuged at 10,000 rpm for 20 mins at 4 °C, and cells were syringe filtered through 45 mm to 0.45 μm nylon membrane filters (Millipore) and adjusted to pH 6.5 using 5 N NaOH to exclude the antimicrobial activity of organic acids. For determining the heat resistance of antimicrobial compound. The CFS was subjected to heat treatment at 80 °C in a water bath for 1 h, then the CFS was immediately cooled by cold water and tested for antimicrobial activity against *S. paratyphi* using 96-well microliter plates. For hydrogen peroxide assay, 5 mL of CFS was treated with 0.5 mg/mL of catalase. To evaluate the stability of the extract during freeze-thaw cycles. The CFS was stored at −20 °C for 24 h and thawed for 10 mins at 5 °C [30]. The 15% of CFS was prepared and poured to 96-well plate, and the 20 μL of *S. paratyphi* were added to each wells containing neutralized CFS then made up the final volume to 200 μL using Luria-Bertani (LB) broth and incubated at 37 °C for 24 h for the antimicrobial activity against *S. paratyphi*. The total percent inhibition of pathogen was calculated using the formula: [(OD of positive control – OD of test samples)/OD of positive control]*100.

3. Results

3.1. Identification and characterization of LAB isolates

All the 130 LAB library isolates were isolated from different traditional fermented foods, whose preliminary characteristics such as catalase, microscopic and their ability to grow on a selective media was studied but no other attributes. Further, in the present study, all the 130 isolates were screened for their potential antibacterial and antifungal activity as preliminary screening, out of which 12 isolates were selected based on their potential antimicrobial activity. Biochemical tests revealed that 12 LAB isolates showed gram-positive, catalase-negative, rod-shaped, Non-motile and absence of endospores. The isolates were able to ferment all the sugars tested with acid production and showed optimal growth at 37 °C after 24 h incubation compared to 4, 10 and 45 °C. Hence it can be concluded that 37 °C is the most ideal for the growth of all strains (Fig. 1). Out of 12 LAB isolates, MYSRD 108 and MYSRD 71 proved their potential probiotics. The results are represented in Table 1.

3.1.1. Antimicrobial activity of LAB isolates

The antibacterial effect of LAB isolates MYSRD 108, and MYSRD 71 was investigated using the microtitre plate assay on five selected enteric pathogens, namely *E. coli, K. pneumonia, S. aureus, P. aeroginosa* and
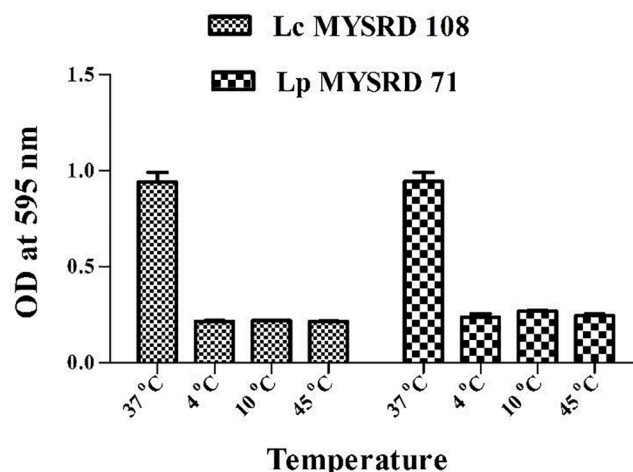

Fig. 1. Growth of probiotic isolates at different temperature.

Table 1
Phenotypic characteristics of LAB isolates.

Test	Isolate MYSRD 108	Isolate MYSRD 71
Gram's staining	Gram Positive	Gram Positive
Shape	Short rods	Short rods
Catalase	Catalase Negative	Catalase Negative
Bile salt hydrolysis (0.3%)	+	+
Growth at different temperature (°C)		
4	+	+
10	+	+
37	++	++
45	+	+
Growth at different pH		
2.0	+++	+++
4.0	+++	+++
6.5	+++	+++
Carbohydrate fermentation test		
Lactose	+	+
Mannitol	+	+
Maltose	+	+
Sucrose	+	+
Arabinose	+	+
Sorbitol	+	+
Antibiotics (mcg)		
Clindamycin (2)	Susceptible	Susceptible
Streptomycin (10)	Susceptible	Susceptible
Vancomycin (30)	Resistant	Resistant
Tetracycline (30)	Susceptible	Susceptible
Chloramphenicol (30)	Susceptible	Susceptible
Erythromycin (15)	Susceptible	Susceptible
Tylosine (15)	Susceptible	Susceptible
Kanamycin (30)	Resistant	Resistant
Gentamycin (10)	Susceptible	Susceptible

*Positive result +(≤ 60%),++ (60% ≥ 70%) and +++ (70% ≥) and - Negative result.

S. paratyphi obtained from ATCC. The two isolates had the strongest antimicrobial activity of 87+1.92%, 81+0.8%, 79+2.9%, 80+0.7% and 81+0.8 % for isolate MYSRD 108 and 88+0.4%, 81+4.6%, 81+2.2%, 81+1.1% and 82+2.1% for isolate MYSRD 71 from 15% concentration of CFS against all the pathogens tested, respectively. Whereas the isolates MYSRD 108 showed moderate activity at 10% concentration of CFS against all the pathogens but showed the highest activity for isolates MYSRD 71 against *S. paratyphi, S. aureus* and *P. aeroginosa* at 10% concentration of CFS (Fig. 2). The activity may be due to the presence of antimicrobial compounds (Bacteriocin, organic acids and hydrogen peroxide) produced by the potential probiotics.

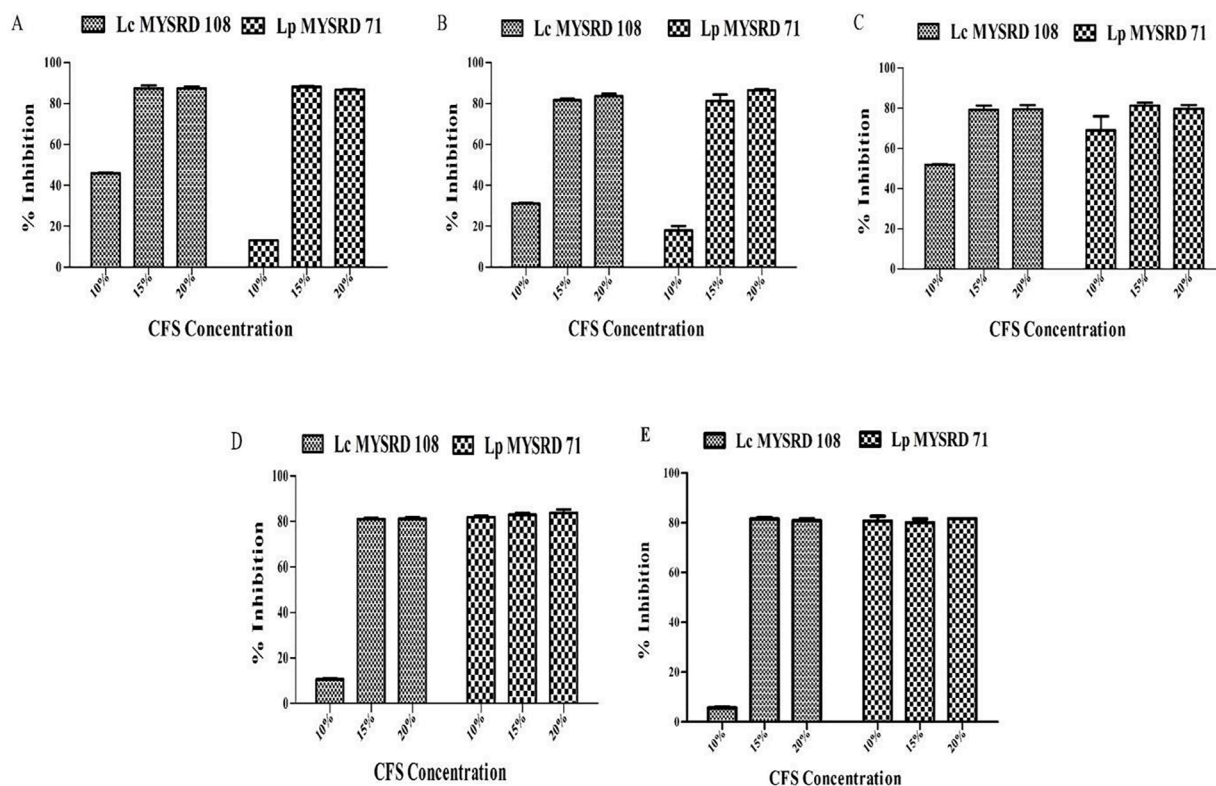LAB isolates MYSRD 108 and MYSRD 71 were also tested for

Fig. 2. Antibacterial activity of LAB cell-free supernatants (of 10, 15 and 20% concentration) against tested enteric pathogens presented as percent inhibition. (A) Inhibition of *Escherichia coli.* (B) Inhibition of *Klebsiella pneumonia.* (C) Inhibition of *Staphylococcus aureus.* (D) Inhibition of *Pseudomonas aeruginosa.* (E) Inhibition of *S. paratyphi.* Data shown are mean ± SD of triplicate values of independent experiments.

antifungal activity against eight fungal species. The LAB isolate MYSRD 71 showed the highest zone of inhibition against all the fungus except *F. graminearum*. Whereas isolates MYSRD 108 had no inhibitory effect after 7 days of incubation against all the fungus tested (Fig. 3). The results are represented in Table 2.

3.1.2. Molecular characterization of LAB isolates

Potential LAB isolates were identified using 16S rRNA gene sequencing. After measuring the quantity of genomic DNA using nanodrop and determining the quality using agarose gel electrophoresis of amplified PCR products, they were blasted and deposited in NCBI at gen bank under Accession number MN907474 and MN907537. The isolates were identified as *L. casei* and *L. plantarum* for MYSRD 108 and MYSRD 71, respectively. The phylogenetic tree was constructed based on the 16S rRNA gene sequences from evolution distance by the neighbour-joining method (Fig. 4).

Table 2

Antifungal activity of LAB strains against fungal species after 7 days of incubation.

Fungal isolates	Lc. MYSRD 108		Lp. MYSRD 71	
	3 days	7 days	3 days	7 days
Fusarium sporotrichiodes	+	–	+++	+++
F. equiseti	+	–	+++	+++
F.avenaceum	+	–	+++	+++
F.graminearum	+	–	++	–
F. poea	+	–	+++	+++
F.verticillioides	+	–	+++	++
Aspergillus parasiticus	+	–	+++	+
Rhizopus oryzae	+	–	+++	+

Diameter of Zone of Inhibition: + diameter of zone of inhibition between 1 and 5 mm, ++ (5 to 15 mm), +++ (15 to 30 mm) and – represents negative result.
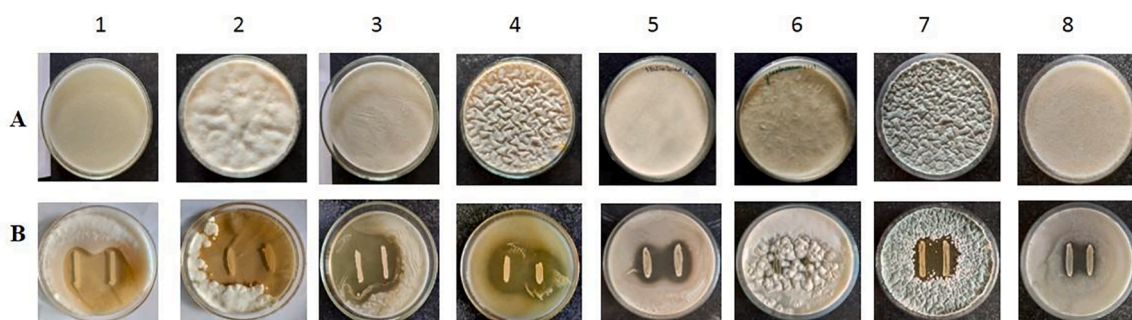

Fig. 3. The antifungal activity of the LAB isolates was tested against 1- *F. Sporotrichioides*, 2- *F. equiseti*, 3- *F-poea*, 4- *F-avenaceum*, 5- *F. verticillioides*, 6-*F. graminearum* 7. *A. parasiticus* and 8. *Rhizopus oryzae*. A represents the fungal control plates after 7 days of incubation, and B represents the antifungal activity of isolate MYSRD 71 after 7 days of incubation against all the fungal species tested.
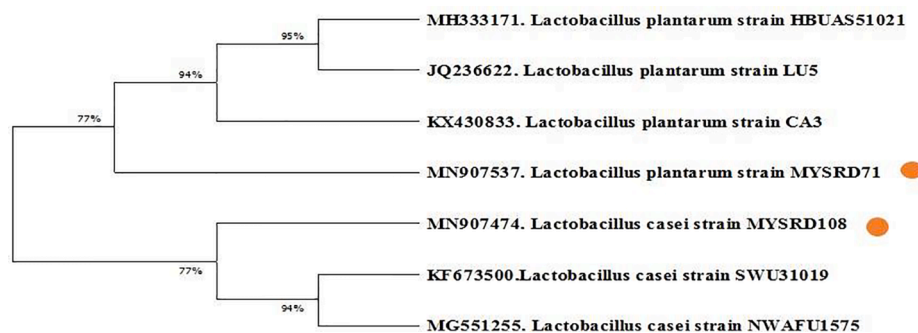
Fig. 4. A neighbor-joining Phylogenetic tree constructed using Mega-X software based on Lactobacillus nucleotide sequences showing the position of *Lactobacillus casei* MYSRD 108 (MN907474) and *Lactobacillus plantarum* MYSRD 71 (MN907537). Book strap values are indicated at the nodes for 500 replicates.

3.2. Evaluation of probiotic properties

3.2.1. Bile and acidic pH tolerance

Fig. 5(A) represents the results of bile tolerance of MYSRD 108 and MYSRD 71 probiotic isolates against 0.3% oxgall for 0–5 h at 37 °C in MRS agar plates. The survival rate was more than 50% for two isolates tested for 5 h incubation. Acidic pH tolerance is one of the major criteria for the validation of probiotics. The probiotic isolates MYSRD 108 and MYSRD 71 showed tolerance to pH 2 and 4 for 0–5 h of incubation at 37 °C in MRS agar plates shown in Fig. 5(B) and (C), respectively. The isolates showed an above 70% survival rate at both pH 2 and pH 4. The survival rate% at pH 2 for 0, 1, 2, 3, 4 and 5 h were 89, 87, 84, 79, 60 and 57% for MYSRD 108 respectively, whereas 84, 82, 80, 71, 66 and 58% for MYSRD 71 respectively. Regarding pH 4 the survival rate% for 5 h were 81, 78, 75, 72, 65 and 54% for MYSRD 108 and 86, 85, 81, 75, 73 and 62% for MYSRD 71 respectively. The viability of the strains decreased as the increase in incubation time when compared to control (pH 6) for 5 h incubation.

3.2.2. Antibiotic susceptibility test

Probiotic strains need to have a safety profile for applications in food and humans. The susceptibility to antibiotics was determined with nine antibiotics using the Kirby Bauer disk diffusion method, and obtained results were compared with a reference chart of Performance standards for antimicrobial disk susceptibility tests. The two isolates were susceptible to Streptomycin, Clindamycin, Tetracycline, Chloramphenicol, Erythromycin, Tylosin and Gentamycin both isolates are resistant to Vancomycin and Kanamycin are shown in Table 1.

3.2.3. Auto-aggregation

The results of the autoaggregation of the probiotic isolates were illustrated in Fig. 6. Among the two LAB isolates tested, MYSRD 108 showed the highest autoaggregation of 89.2% after 5 h of incubation, followed by isolates MYSRD 71 showed 88.5%, respectively.

3.2.4. Cell surface hydrophobicity

The percentage of cell surface hydrophobicity for 5 h of incubation at
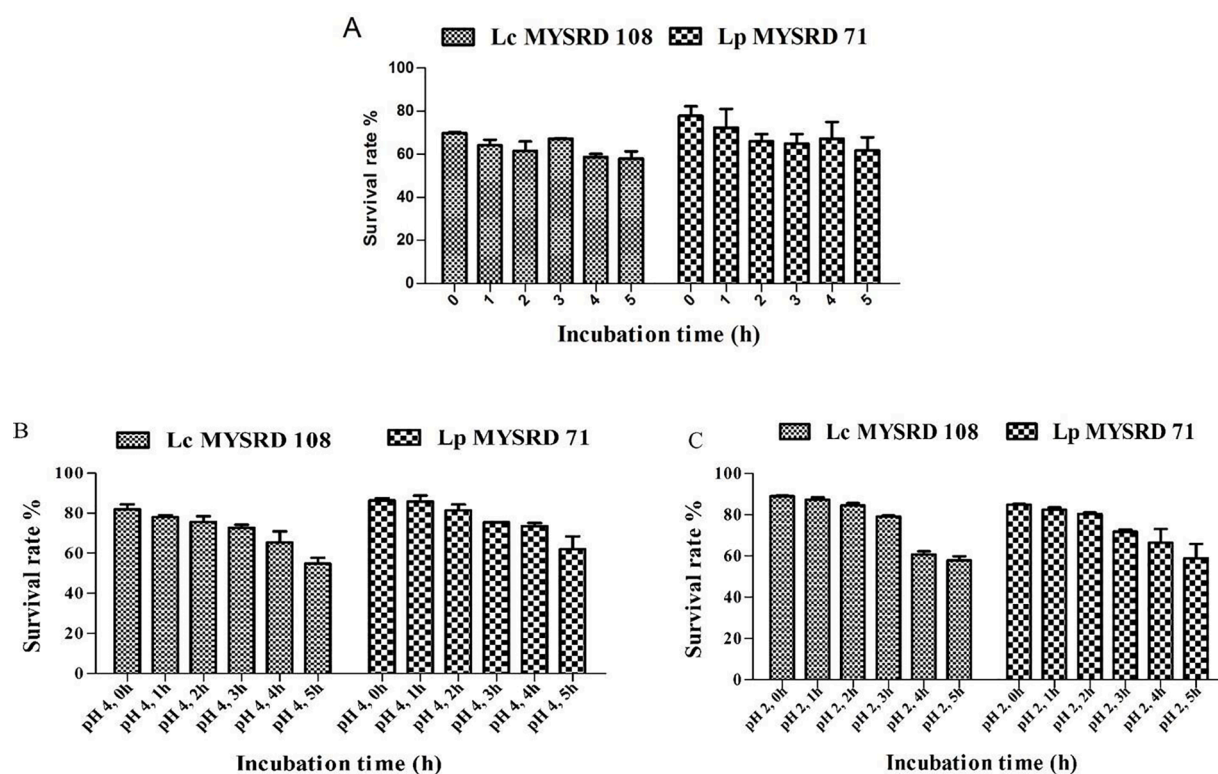

Fig. 5. Bile and acid tolerance of LAB strains A) Survival of the isolates under 0.3% bile salt conditions for 0, 1, 2, 3, 4 and 5 h at 37 °C in MRS agar plates. (B) Survival of the isolates under acidic pH 4 for 0, 1, 2, 3, 4 and 5 h at 37 °C in MRS agar plates. (C) Survival of the isolates under acidic pH 2 for 0, 1, 2, 3, 4 and 5 h at 37 °C in MRS agar plates. Data shown are mean ± SD of triplicate values of independent experiments.
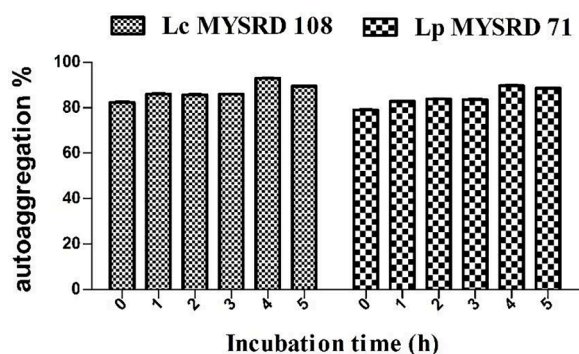
Fig. 6. Auto-aggregation of LAB strains evaluated for 5 h at 37 °C. The data shown represents the standard deviation of the mean value of results in error bars.

37 °C against hydrocarbon, namely xylene were presented in Fig. 7. Among the two isolates investigated, MYSRD 71 showed a higher percentage of cell surface hydrophobicity of 64% after 5 h of incubation, followed by MYSRD 108 of 61%, respectively. This indicates that LAB isolates tested have an adhering capacity to epithelial cells and mucosal surfaces.

3.3. Evaluation of anti-salmonella attributes

3.3.1. Determination of the antimicrobial activity of selected LAB isolates using agar well diffusion

The antimicrobial effect of LAB isolates obtained from traditional fermented food Vellappam was assayed against *S. paratypi* using the agar well diffusion test. All 12 strains of LAB CFS were tested against *S. paratyphi*, *L. casei* MYSRD 108 showed the highest inhibition from CFS of 50 to 500 μL/well followed by *L. plantarum* MYSRD 71 from CFS of 200 μL/well respectively. Whereas on the other hand, a variable degree of antagonism against *S. paratyphi* was found, as seen in (Table 3). Other ten isolates showed a zone of inhibition around the wells provided with 500 μL of the LAB CFS with the zone of inhibition between 17 and 20 mm in diameter.

3.3.2. Determination of MIC using the microtitre plate method

The antimicrobial activity of *L. casei* MYSRD 108 and *L. plantarum* MYSRD 71 was determined using the broth microdilution method against *S. paratyphi* (Fig. 8). The results indicated that *L. planatrum*

MYSRD 71 showed antibacterial activity from 12% concentration of CFS followed by *L. casei* MYSRD 108 with antibacterial activity from 15% concentration of CFS with a percentage inhibition of 80 and 81%, respectively.

3.3.3. In-vitro time-kill assay

In-vitro time-kill assay evaluated the cell count reduction of *S.paratyphi* in the presence of CFS of *L. casei* MYSRD 108 and *L. plantarum* MYSRD 71 with the different incubation periods of 0, 3, 6, 12, 15, 18, 21 and 24 h. The MYSRD 71 completely inhibited the pathogen after 18 h of incubation, followed by MYSRD 108 with the reduction in pathogen colonies when compared to control, as shown in Fig. 9.

3.3.4. Lactobacillus and salmonella co-culture experiment

The ability of *L.casei* MYSRD 108 and *L. plantarum* MYSRD 71 to inhibit the growth of *S. paratyphi* in-vitro was evaluated in a co-culture experiment. The two *Lactobacillus* inhibited the growth of *S. paratyphi* dramatically after 24 h of incubation at 37 °C. The results represented in Fig. 10

3.3.5. Biofilm assay

The biofilm-forming ability of probiotics (*L. casei* MYSRD 108 and *L. plantarum* MYSRD 71) and *S. paratyphi*, were demonstrated in 96-well microlitre plate using crystal violet method. Both probiotic were strains dependent and biofilm producers in MRS broth. The highest O.D of 1.92 (*L. casei* MYSRD 108) and 1.62 (*L. plantarum* MYSRD 71) were observed. The values for the pathogen *S. paratyphi* were under 1, i.e. 0.72 O.D but were strong biofilm producers.

3.3.6. Biofilm inhibition assay using crystal violet method

The biofilm-forming potential of *S. paratyphi* ATCC 9150 was determined in the presence of potential probiotics of *L. plantarum* MYSRD 71 and *L. casei* MYSRD 108 with cell-free supernatant (15% CFS) as shown in Fig. 11. The 15% CFS of two Lactobacillus (*L. casei* and *L. plantarum*) resulted in 75 and 81% inhibition of the *S. paratyphi* biofilm formation, respectively. The *S. paratyphi* in the presence of CFS of LAB after 24 h was significantly reduced compared to the biofilm formation of *S. paratyphi* alone (Positive control).

3.3.7. Biofilm inhibition assay using MTT

The effect of *L. plantarum* MYSRD 71 and *L. casei* MYSRD 108 CFS on the biofilm-forming ability of *S. paratyphi* was determined by MTT reduction assay in a 96 well plate. In this MTT reduction assay. The
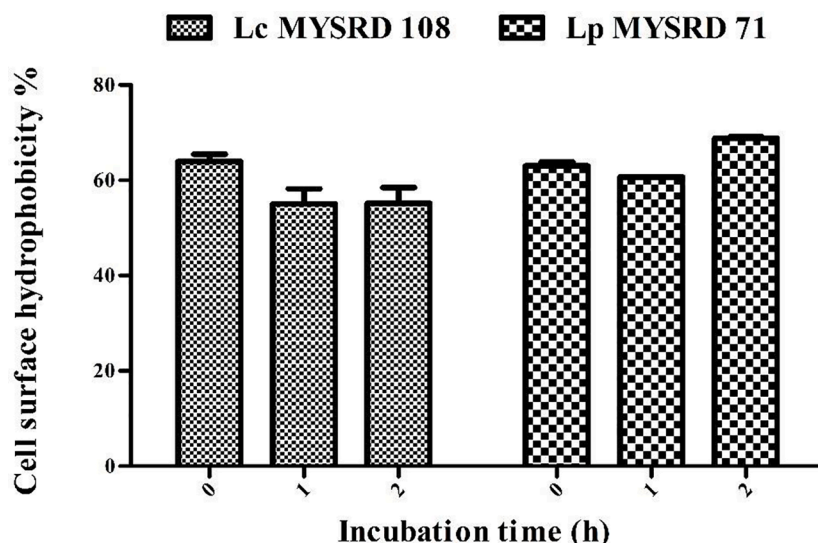

Fig. 7. Cell surface hydrophobicity of LAB isolates evaluated for 5 h at 37 °C. Data shown represents the standard deviation of the mean value of results in error bars.

Table 3

Antimicrobial activity of cell-free supernatant of selected isolates using agar well diffusion assay against *S. paratyphi*.

LAB - Cell free supernatants (from 25 to 500 μL/well)	Isolate MYSRD 108	Isolate MYSRD 71	Isolate MYSRD S1	Isolate MYSRD S4	Isolate MYSRD 67	Isolate MYSRD 78	Isolate MYSRD 58	Isolate MYSRD 57	Isolate MYSRD 80	Isolate MYSRD 126	Isolate MYSRD 113	Isolate MYSRD 21
25	–	–	–	–	–	–	–	–	–	–	–	–
50	18.5±0.7	–	–	–	–	–	–	–	–	–	–	–
75	18.6±0.5	–	–	–	–	–	–	–	–	–	–	–
100	18.5±0.2	–	–	–	–	–	–	–	–	–	–	–
125	18.8±0.9	–	–	–	–	–	–	–	–	–	–	–
150	18.8±0.9	–	–	–	–	–	–	–	–	–	–	–
175	19.8±0.2	–	–	–	–	–	–	–	–	–	–	–
200	19.5±0.5	17.5±0.7	–	–	–	–	–	–	–	–	–	–
225	19.5±0.5	17.7±0.3	–	14.6±0.5	–	–	–	–	–	–	–	–
250	19.5±0.5	18.5±0.7	–	14.5±0.2	–	–	–	–	–	–	–	–
275	19.8±0.9	18.5±0.7	–	13.8±0.9	–	–	–	–	–	–	–	–
300	20.5±0.7	19.0±0.0	–	18.9±0.9	–	–	–	–	–	–	–	–
325	20.5±0.5	20.5±0.7	20.7±0.3	18.0±0.0	19.5±0.7	–	–	–	–	19.5±0.7	–	16.7±0.3
500	20.7±0.3	20.7±0.3	20.8±0.9	20.0±0.0	20.0±0.0	19.5±0.5	18.0±0.0	19.5±0.7	18.0±0.0	20.7±0.3	19.0±0.0	18.0±0.0

The diameter of Zone of Inhibition measured in mm.Each valve in the table is mean+ standard deviation of triplicates
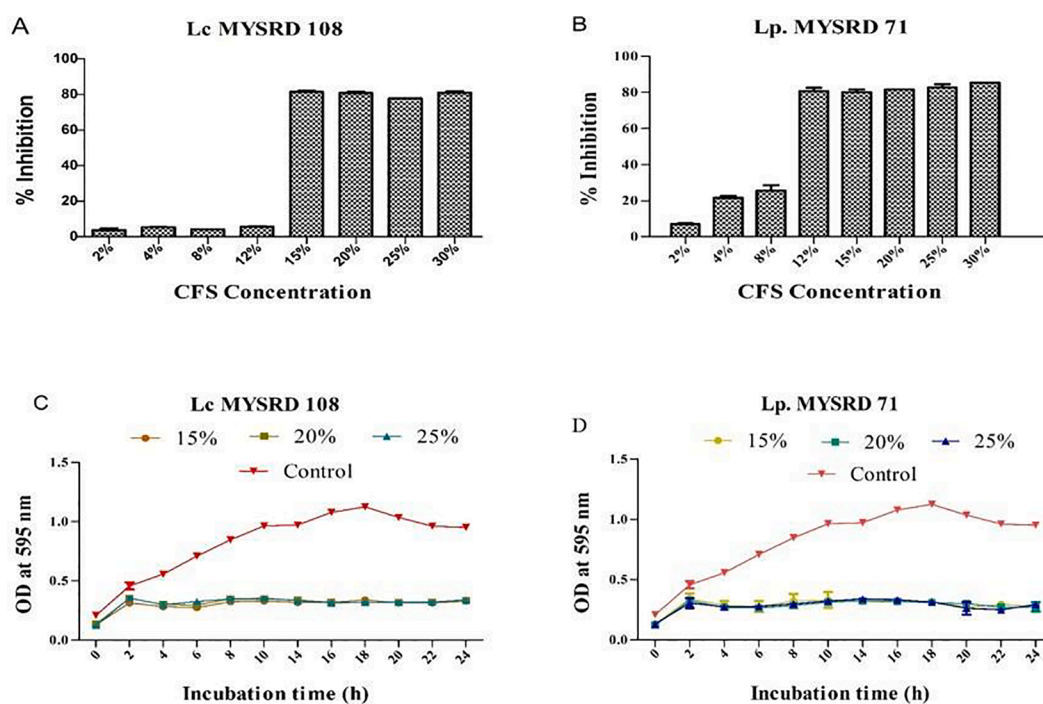

Fig. 8. Minimum Inhibitory Concentration of *S. paratyphi* with various concentrations of Cell-free supernatant (A) Percentage Inhibition of *S. paratyphi* by cell-free supernatant of *L. casei* MYSRD 108. (B) Percentage Inhibition of *S. paratyphi* by cell-free supernatant of *L. plantarum* MYSRD 71. (C) *S. paratyphi* growth inhibition by cell-free supernatant of *L. casei* MYSRD 108. (D) *S. paratyphi* growth inhibition by cell-free supernatant of *L. plantarum* MYSRD 71.

S. paratyphi were significantly reduced in the presence of LAB-CFS at the lower concentration of 15% with the strongest inhibition of 79% and 77% in the case of *L. casei* and *L. plantarum,* respectively, as shown in Fig. 12. The reduction of MTT showing purple color observed in the positive control is due to the activity of metabolic enzymes present in the live cells of *S. paratyphi*.

3.3.8. SEM analysis

The antibacterial effect of LAB-CFS on *S. paratyphi* was observed by SEM (Fig. 13(B and C)). The treated *S. paratyphi* with CFS of LAB had a shriveled retracted appearance and found some alterations in structure and membrane of *S. paratyphi* cells when compared to untreated *S. paratyphi,* i.e. control (Fig. 13(A)) had a uniform, commitment and well-developed biofilms.

3.4. Characterization of the antimicrobial substances

The anti-salmonella activities of the cell-free supernatant of *L. casei* MYSRD 108 and *L. plantarum* MYSRD 71 were determined by neutralizing the CFS to 6.5 with 5 M NaOH. The antimicrobial activities of CFS after neutralizing to pH 6.5 against *S. paratyphi* were completely abrogated. Further, heat-treated CFS (CFS + Heat), hydrogen peroxide test (CFS + H_2O_2), un-neutralized CFS and stability during storage at −20 °C (CFS - 20 °C) showed maximal activity of above 60 and 65% for *L. casei* MYSRD 108 and *L. plantarum* MYSRD 71 respectively against *S. paratyphi* tested proving the role of the organic acid for their antimicrobial activity (Fig. 14).
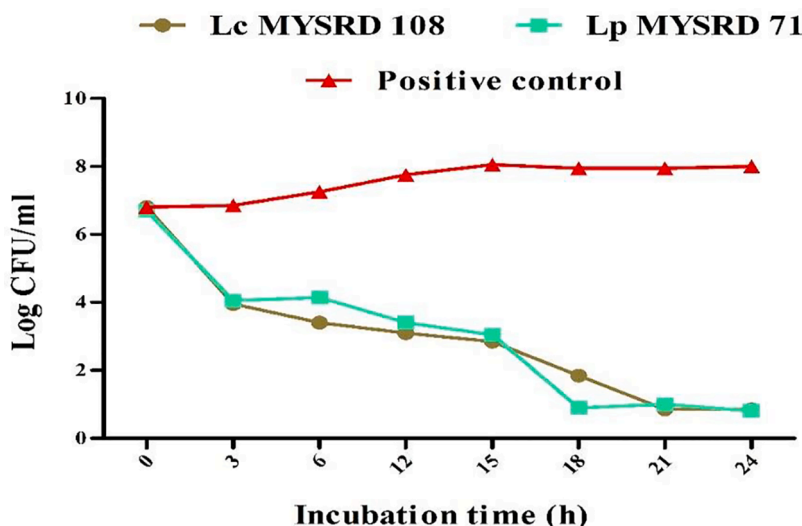
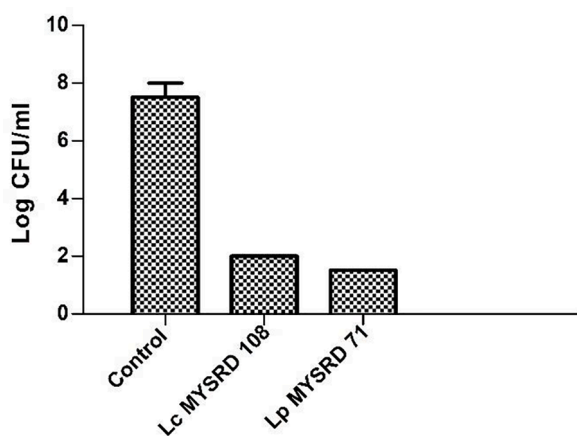**Fig. 9.** Evaluation of LAB-CFS on *S. paratyphi* by *in-vitro* time-kill assay.

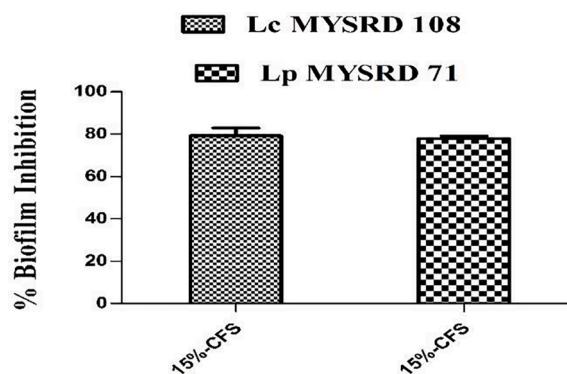**Fig. 10.** Reduction in the growth of *S. paratyphi* in the presence of LAB strains.

Fig. 11. Biofilm inhibition of *S. paratyphi* with probiotics CFS by crystal violet assay. Percentage inhibition of *S. paratyphi* biofilms from probiotics cell-free supernatant of 15%. Error bars are representative of the standard deviation and mean.

Fig. 12. Biofilm inhibition of *S. paratyphi* with probiotics CFS by MTT assay. (A) Percentage inhibition of *S. paratyphi* biofilms from probiotics cell-free supernatant at 15% by MTT assay. Error bars are representative of the standard deviation and mean.
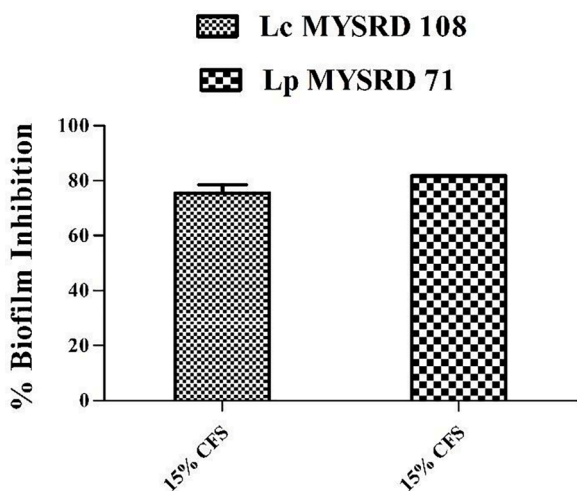
4. Discussion

In the present study, we identified two potential probiotic isolates, MYSRD 108 and MYSRD 71 as L.*casei*, and L. *plantarum,* respectively. Various LAB isolated from traditional fermented food such as L. *casei* and L. *planatrum* [31] has been reported to have probiotic activity. Resistance to bile salts and acidity are important criteria for the selection of potential probiotics, as LAB strains ingested need to survive in the bile salts in the intestine and survive to the acid condition of the stomach for bacterial colonization in the host. Hence the selection of probiotics strains with high resistance to bile salts and high tolerance to acidic conditions is essential so they could colonize better in the upper intestine [32]. Therefore, in our experiment, we observed the growth of both LAB isolates in various acidic pH values (2, 4 and 6.5) and additionally survived in the presence of bile salt at 0.3% oxgall, where 0.3% bile is the maximum concentration present in human [33]. Accordingly, these isolates may survive in the gastrointestinal conditions and colonize in the large intestine. Antibiotics are utilized by the medical industries as a major tool to fight against various pathogens, but their resistance to common pathogens can cause a significant threat to the treatment of nosocomial and community-acquired infections [34]. Therefore, the evaluation of the antimicrobial resistance of Lactobacillus strains is an important criterion for the selection of potential probiotics because the commercial use of probiotics encompassing antibiotic-resistant genes
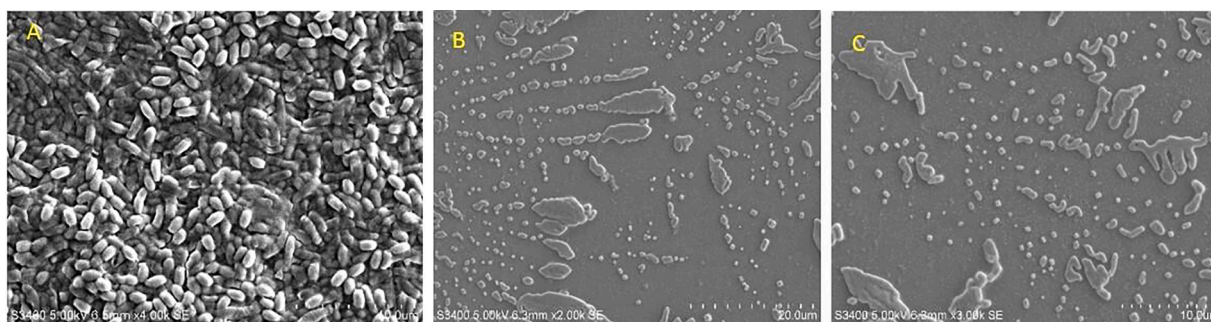
Fig. 13. Scanning electron microscopy of the effect of cell-free supernatant from probiotics on *S. paratyphi* biofilms (A) SEM of *S. paratyphi* ATCC 9150 (B) *S. paratyphi* treated with L. *casie* MYSRD 108 (C) *S. paratyphi* treated with L. *plantarum* MYSRD 71.
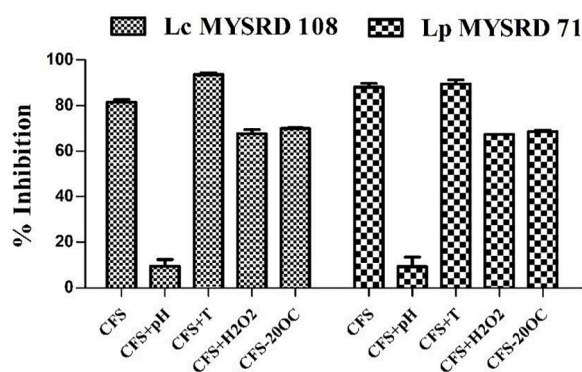

Fig. 14. Antimicrobial activity of neutralized cell-free supernatant of L. *casie* MYSRD 108 and L. *plantarum* MYSRD 71 against *S. paratyphi*. Note: CFS: Cell free supernatant, CFS+ pH: CFS on treatment with pH 6.5, CFS+*H*: CFS on treatment with Heat, CFS+H$_2$0$_2$: CFS on treatment with hydrogen peroxide and CFS-20 °C: CFS stability during storage at −20 °C.

could be transferred to the pathogens present in the intestine [35]. We evaluated the safety of the two strains by determining their susceptibility to nine antibiotics, but the strains L. *casei* MYSRD 108 and L. *plantarum* MYSRD 71 were resistant only to two antibiotics, vancomycin and kanamycin, which is similar to other studies reported that many species of *Lactobacillus* have a high natural resistance to vancomycin [36] and kanamycin [37]. The adhesion of probiotic bacteria to the mucosal surfaces is essential for the competitive elimination of pathogenic microorganisms in the intestine [38]. Cell surface properties tested by autoaggregation and cell surface hydrophobicity are analytic parameters for potential probiotic cell adhesion to epithelial cells in the human intestine. In our study, LAB isolates, however, exhibited comparatively higher autoaggregation of 89.2% for MYSRD 108 and 88.5% for MYSRD 71 and Hydrophobicity of 64% for MYSRD 71 and 61% for MYSRD 108 as compared with the previous study reported by Somashekaraiah et al. [18]. with hydrophobicity ranging 50 to 77.82% and autoaggregation ranging from 40 to 78.95% from traditional fermented food –Neera. Our study further revealed that two LAB isolates grew optimally at 37 °C after 24 h incubation, and growth was reduced at 4 °C, 10 °C and 55 °C. This finding is similar to the previous report [39].

Probiotics, when administered in adequate amounts, confer health benefits to the host [40]. Hence, an important beneficial effect of probiotics is antimicrobial activity against pathogens [41]. The antagonistic activity of L. *Casei* MYSRD 108 and L. *plantarum* MYSRD 71 showed the highest antibacterial activity against the enteric pathogens tested at 15% CFS against *E. coli* (ATCC 25,922), *K. pneumoniae* (MTCC 7407), *P.aeruginosa* (ATCC 15,422) and *S. aureus* (ACTT 6538). Cell-free supernatant of human L. *acidophilus* strain LB decreased the viability of *Shigella flexneri, E. coli, K. pneumoniae, S. aureus,* L. *monocytogenes, S.*

typhimurium, B. cereus, P. aeruginosa, and *Enterobacter* spp [42]. Moreover, The L. *plantarum* MYSRD 71 also showed the strongest antifungal activity for all the *Fusarium* tested except *F. gramenearum*. Also, several studies have been reported on the production of antimicrobial compounds by L. *plantarum* strain with antifungal activity [43].

Among food-borne pathogens, *Salmonella* plays the main role in food microbiology [44]. The antimicrobial activity of the cell-free culture supernatant of twelve *Lactobacillus* isolates against *S. paratyphi* was evaluated by agar well diffusion and resulted in strong activity (inhibition zone ≥ 18 mm) for MYSRD 108 and MYSRD 71 with the wells provided with CFS of 50 μL and 200 μL respectively. Findings are in agreement with Daim et al. [45], where among 32 *Lactobacillus* isolates, thirteen isolates showed relatively strong activity (inhibition zone ≥ 15 mm) and seven isolates showed moderate activity (inhibition zone < 15–10 mm) against *S. typhi*. Further, different concentrations (2% −50%) of CFS of MYSRD 108 and MYSRD 71 were used to study its effect on the viability of *S. paratyphi* using the broth microdilution method in a 96-well microtitre plate. The inhibition of *S. paratyphi* increased with increasing concentration of the CFS and found 80% inhibition with 12% of CFS and 81% inhibition with 15% of CFS for MYSRD 71 and MYSRD 108, respectively. The previous study has reported that effective killing of *S. enteritidis* (89.6%) with 11% of CFS after 4 h [46].

In the present study, the high interference of two *Lactobacillus* isolates (MYSRD 108 and MYSRD 71) with the invasion of *S. paratyphi* was exhibited strong antimicrobial activity in the co-culture experiment. Therefore, the valuation of interaction between a *Lactobacillus* and *S. paratyphi* can be obtained when they are cultured in the same medium (MRS-MH) and by sharing the same environmental growth conditions [45]. Our obtained results revealed that two *Lactobacillus* isolates, MYSRD 108 and MYSRD 71, inhibited the growth of *S. paratyphi* to undetectable levels after 24 h of incubation at 37 °C. In agreement with our findings, Fayol-Messaoudi et al. [47]. investigated the L. *plantarum* strain ACA-DC287 determined that the co-culture with *S. typhimurium* resulted in the killing of the pathogen. Further, time kills assay resulted in the reduction of cell counts of *S. paratyphi* with the presence of L. *casei* MYSRD 108 and L. *plantarum* MYSRD 71, with the increase in the incubation period. The findings are similar to the Prabhurajeshwar and Chandrakanth [48].

The formation of pathogenic biofilms is a life-threatening issue in clinical fields like nosocomial infections and in food processing. The *Lactobacillus* plays a major role in reducing the pathogenic biofilms [49]. The biofilm formation of LAB strains with the highest OD of 1.92 for L. *casei* MYSRD 108 and 1.62 for L. *plantarum* MYSRD 71 and OD of 0.72 for *S. paratyphi* with strong biofilm producers obtained in the present study showed similar results with OD 1.65 for L. *lactis* 368 and 1.38 for L. *helveticus* 352 [50]. The obtained result from the crystal violet method showed a maximum reduction of 75% and 81% of *S. paratyphi* biofilms by CFS of L. *plantarum* MYSRD 71 and L.*casei* MYSRD 108, respectively. In line with our study, Woo and Ahn [51] obtained similar results against *Salmonella* and *Listeria monocytogenes*.

The antibiofilm activity of LAB strains on *S. paratyphi* was evaluated using MTT assay in a 96-well microtitre plate. Liu et al. [52] reported the biofilm inhibitory effects of the purified Exo Polysaccharides(EPS) of 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL on 4 pathogens (i.e., *P. aeruginosa* CMCC10104, E. coli O157:H7, *S. typhimurium* ATCC13311, and S. *aureus* CMCC26003) increased gradually with increasing concentrations of EPS (0.2 to 5.0 mg/mL), and the highest inhibition observed for *P. aeruginosa* CMCC10104 and *S. typhimurium* ATCC13311 (47.02 ± 4.83%) and lowest for *E. coli* O157:H7 (25.82 ± 5.34%). But in our study observed the strongest biofilm inhibition of *S. paratyphi* of about 79% and 77% for L. *casei* MYSRD 108 and L. *plantarum* MYSRD 71, respectively.

The mechanism of *Lactobacillus* spp can work as a microbial barrier against intestinal pathogen through modulation of host's immune system, competitive exclusion of pathogen binding; another essential condition is antimicrobial compounds production such as organic acids (e. g., lactic acid, acetic acid, propionic acid) and proteinaceous compounds such as bacteriocins to antagonize pathogens [53,54]. Therefore the CFS of LAB isolates were screened against the food-borne pathogen *S. paratyphi* for their antimicrobial property. In our study, the anti-salmonella activities of neutralized cell-free supernatant of LAB may often be due to the production of organic acids after conducting the experiments for the production of antimicrobial compounds such as hydrogen peroxide assay, storage stability at −20 °C and heat treatment no bacteriocin-like inhibitory substance was detected. Jin Lizhi [55] reported that the inhibitory effect of *Lactobacillus* strains against *Salmonella* and *E.coli* might often be due to the production of organic acids.

5. Conclusion

In conclusion, potential probiotics were successfully isolated and identified as L. *casei* MYSRD 108 and L. *plantarum* MYSRD 71 strain that exhibited intensively probiotic properties such as strong survival in acidic pH conditions, bile tolerance, auto aggregation, cell surface properties and were susceptible to a numbers of clinically effective antibiotics. Moreover, two isolates showed broad spectrum of antagonistic activities for probiotic application in the gastrointestinal tract against food-borne pathogen *S. paratyphi*. Therefore the production of antimicrobial compounds in the cell-free supernatant has great probiotic potential. However, further research is needed to evaluate them by *in-vivo* assay in animal experiments.

CRediT authorship contribution statement

S. Divyashree: Conceptualization, Data curation, Formal analysis, Writing – original draft. **P.G. Anjali:** Data curation, Formal analysis. **Rakesh Somashekaraiah:** Project administration, Funding acquisition. **M.Y. Sreenivasa:** Conceptualization, Project administration, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgement

References

[1] G. Midelet, B. Carpentier, Impact of cleaning and disinfection agents on biofilm structure and on microbial transfer to a solid model food, J. Appl. Microbiol. (2004) 262–270, https://doi.org/10.1111/j.1365-2672.2004.02296.x.

[2] J. Schnürer, J. Magnusson, Antifungal lactic acid bacteria as biopreservatives, Trends Food Sci. Technol. 16 (2005) 70–78, https://doi.org/10.1016/j.tifs.2004.02.014.

[3] A. Fàbrega, J. Vila, Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation, Clin. Microbiol. Rev. 26 (2013) 308–341, https://doi.org/10.1128/CMR.00066-12.

[4] E. Giaouris, E. Heir, M. Desvaux, M. Hébraud, T. Møretrø, S. Langsrud, A. Doulgeraki, G.J. Nychas, M. Kacániová, K. Czaczyk, H. Ölmez, M. Simões, Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens, Front. Microbiol. 6 (2015) 1–26, https://doi.org/10.3389/fmicb.2015.00841.

[5] P.G. Pappas, Antifungal clinical trials and guidelines: what we know and do not know, Cold Spring Harb, Perspect. Med. 4 (2014), https://doi.org/10.1101/cshperspect.a019745.

[6] D.V.T. Nair, K. Venkitanarayanan, A.K. Johny, Antibiotic-resistant Salmonella in the food supply and the potential role of antibiotic alternatives for control, Foods 7 (2018), https://doi.org/10.3390/foods7100167.

[7] T.S. Desin, W. Köster, A.A. Potter, Salmonella vaccines in poultry: past, present and future, Expert Rev. Vaccines. 12 (2013) 87–96, https://doi.org/10.1586/erv.12.138.

[8] J.E. Nett, Future directions for anti-biofilm therapeutics targeting Candida, Expert Rev. Anti. Infect. Ther. 12 (2014) 375–382, https://doi.org/10.1586/14787210.2014.885838.

[9] M.E. Hibbing, C. Fuqua, M.R. Parsek, S.B. Peterson, Bacterial competition: surviving and thriving in the microbial jungle, Nat. Rev. Microbiol. 8 (2010) 15–25, https://doi.org/10.1038/nrmicro2259.

[10] R.A. Britton, J. Versalovic, Probiotics and gastrointestinal infections, Interdiscip. Perspect. Infect. Dis. 2008 (2008) 1–10, https://doi.org/10.1155/2008/290769.

[11] Y.Z. Nur, B. Aslim, Assessment of potential probiotic and starter properties of Pediococcus spp. isolated from Turkish-type fermented sausages (Sucuk), J. Microbiol. Biotechnol. 20 (2010) 161–168, https://doi.org/10.4014/jmb.0904.04019.

[12] C.L. Gerez, M.I. Torino, G. Rollán, G. Font de Valdez, Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties, Food Control 20 (2009) 144–148, https://doi.org/10.1016/j.foodcont.2008.03.005.

[13] K. Ström, J. Schnürer, P. Melin, Co-cultivation of antifungal lactobacillus plantarum MiLAB 393 and aspergillus nidulans, evaluation of effects on fungal growth and protein expression, FEMS Microbiol. Lett. 246 (2005) 119–124, https://doi.org/10.1016/j.femsle.2005.03.047.

[14] A. Jamwal, K. Sharma, R. Chauhan, S. Bansal, G. Goel, Evaluation of commercial probiotic lactic cultures against biofilm formation by Cronobacter sakazakii, Intest. Res 17 (2019) 192–201, https://doi.org/10.5217/ir.2018.00106.

[15] J. Magnusson, J. Schnürer, Lactobacillus coryniformis subsp. coryniformis strain Si3 produces a broad-spectrum proteinaceous antifungal compound, Appl. Environ. Microbiol. 67 (2001) 1–5, https://doi.org/10.1128/AEM.67.1.1-5.2001.

[16] U. Edwards, T. Rogall, H. Blöcker, M. Emde, E.C. Böttger, Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA, Nucleic Acids Res 17 (1989) 7843–7853, https://doi.org/10.1093/nar/17.19.7843.

[17] D.J. Lane, B. Pace, G.J. Olsen, D.A. Stahl, M.L. Sogin, N.R. Pace, Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 6955–6959, https://doi.org/10.1073/pnas.82.20.6955.

[18] R. Somashekaraiah, B. Shruthi, B.V. Deepthi, M.Y. Sreenivasa, Probiotic properties of lactic acid bacteria isolated from neera : a naturally fermenting coconut palm nectar, Front. Microbiol. 10 (2019) 1–11, https://doi.org/10.3389/fmicb.2019.01382.

[19] B.V. Deepthi, K.P. Rao, G. Chennapa, M.K. Naik, K.T. Chandrashekara, Antifungal attributes of lactobacillus plantarum mys6 against fumonisin producing fusarium proliferatum associated with poultry feeds, PLoS ONE. (2016), pp. 1–22. 10.1371/journal.pone.0155122.

[20] K. Angmo, A. Kumari, T.C.Bhalla Savitri, Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh, LWT - Food Sci. Technol. 66 (2016) 428–435, https://doi.org/10.1016/j.lwt.2015.10.057.

[21] H. Lee, H. Yoon, Y. Ji, H. Kim, H. Park, J. Lee, H. Shin, W. Holzapfel, Functional properties of lactobacillus strains isolated from kimchi, Int. J. Food Microbiol. 145 (2011) 155–161, https://doi.org/10.1016/j.ijfoodmicro.2010.12.003.

[22] D. Mohanty, P. Ray, Evaluation of probiotic and antimicrobial properties of lactobacillus strains isolated from dairy products, Int. J. Pharm. Pharm. Sci. 8 (2016) 230–234, https://doi.org/10.22159/ijpps.2016v8i11.12683.

[23] H.E. Xinlong, Y. Zou, Y. Cho, Effects of bile salt deconjugation by probiotic strains on the survival of antibiotic-resistant foodborne pathogens under simulated gastric conditions, J. Food Prot. 75 (2012) 1090–1098, https://doi.org/10.4315/0362-028X.JFP-l.

[24] A. Adetoye, E. Pinloche, B.A. Adeniyi, F.A. Ayeni, Characterization and anti-salmonella activities of lactic acid bacteria isolated from cattle faeces, BMC Microbiol. (2018), pp. 1–11. 10.1186/s12866-018-1248-y.

[25] S. Borges, J. Silva, P. Teixeira, Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs, Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 101 (2012) 677–682, https://doi.org/10.1007/s10482-011-9666-y.

[26] D. Sharma, B.S. Saharan, N. Chauhan, S. Procha, S. Lal, Isolation and functional characterization of novel biosurfactant produced by Enterococcus faecium, Springerplus 4 (2015) 1–14, https://doi.org/10.1186/2193-1801-4-4.

[27] N.M. Wijesundara, H.P.V. Rupasinghe, Essential oils from Origanum vulgare and Salvia officinalis exhibit antibacterial and anti-biofilm activities against Streptococcus pyogenes, Microb. Pathog. 117 (2018) 118–127, https://doi.org/10.1016/j.micpath.2018.02.026.

[28] M. Besser, J. Terberger, L. Weber, B. Ghebremedhin, E.A. Naumova, W.H. Arnold, E.K. Stuermer, Impact of probiotics on pathogen survival in an innovative human plasma biofilm model (hpBIOM), J. Transl. Med. 17 (2019) 1–9, https://doi.org/10.1186/s12967-019-1990-4.

[29] P. Shokryazdan, C.C. Sieo, R. Kalavathy, J.B. Liang, N.B. Alitheen, M.F. Jahromi, Y. W. Ho, Probiotic potential of lactobacillus strains with antimicrobial activity against some human pathogenic strains, Biomed Res Int (2014), https://doi.org/10.1155/2014/927268.

[30] A.G. Ponce, M.R. Moreira, C.E. Valle, S.I. Roura, Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables, LWT-Food Sci.Technol 41 (2008) 432–441, https://doi.org/10.1016/j.lwt.2007.03.021.

[31] M.S. Bin Masalam, A. Bahieldin, M.G. Alharbi, S. Al-Masaudi, S.K. Al-Jaouni, S. M. Harakeh, R.R. Al-Hindi, Isolation, Molecular Characterization and Probiotic Potential of Lactic Acid Bacteria in Saudi Raw and Fermented Milk, Evidence-Based Complement, Altern. Med. 2018 (2018), https://doi.org/10.1155/2018/7970463.

[32] S.E. Gilliland, T.E. Staley, L.J. Bush, Importance of Bile Tolerance of Lactobacillus acidophilus Used as a Dietary Adjunct, J. Dairy Sci. 67 (1984) 3045–3051, https://doi.org/10.3168/jds.S0022-0302(84)81670-7.

[33] M. Haddhadin, S.S. Awaisheh, R.K. Robinson, The production of yoghurt with probiotic bacteria isolated from infants in jordan, Pakistan J. Nutr. 3 (2004) 290–293, https://doi.org/10.3923/pjn.2004.290.293.

[34] R.C. Moellering, J.R. Graybill, J.E. McGowan, L. Corey, Antimicrobial resistance prevention initiative-an update: proceedings of an expert panel on resistance, Am. J. Med. 120 (2007) 4–25, https://doi.org/10.1016/j.amjmed.2007.04.001.

[35] S. Mathur, R. Singh, Antibiotic resistance in food lactic acid bacteria - A review, Int. J. Food Microbiol. 105 (2005) 281–295, https://doi.org/10.1016/j.ijfoodmicro.2005.03.008.

[36] B.D. Ayodeji, C. Piccirillo, V. Ferraro, P.R. Moreira, A.O. Obadina, L.O. Sanni, M.M. E. Pintado, Screening and molecular identification of lactic acid bacteria from gari and fufu and gari effluents, Ann. Microbiol. 67 (2017) 123–133, https://doi.org/10.1007/s13213-016-1243-1.

[37] M. Bernardeau, J.P. Vernoux, S. Henri-Dubernet, M. Guéguen, Safety assessment of dairy microorganisms: the Lactobacillus genus, Int. J. Food Microbiol. 126 (2008) 278–285, https://doi.org/10.1016/j.ijfoodmicro.2007.08.015.

[38] A.C. Ouwehand, P.V. Kirjavainen, C. Shortt, S. Salminen, Probiotics: mechanisms and established effects, Int. Dairy J. 9 (1999) 43–52, https://doi.org/10.1016/S0958-6946(99)00043-6.

[39] C. Ibourahema, R.D. Dauphin, D. Jacqueline, P. Thonart, Characterization of lactic acid bacteria isolated from poultry farms in Senegal, African J. Biotechnol. 7 (2008) 2006–2012, https://doi.org/10.5897/ajb2008.000-5048.

[40] FAO/WHO, Guidelines for the evaluation of probiotics in food, (2002) pp. 1–11.

[41] J.M. Laparra, Y. Sanz, Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium, Lett. Appl. Microbiol. 49 (2009) 695–701, https://doi.org/10.1111/j.1472-765X.2009.02729.x.

[42] M.H. Coconnier, V. Liévin, M.F. Bernet-Camard, S. Hudault, A.L. Servin, Antibacterial effect of the adhering human lactobacillus acidophilus strain LB, Antimicrob. Agents Chemother. 41 (1997) 1046–1052, https://doi.org/10.1128/aac.41.5.1046.

[43] H.K. Wang, Y.H. Yan, J.M. Wang, H.P. Zhang, W. Qi, Production and characterization of antifungal compounds produced by Lactobacillus plantarum IMAU10014, PLoS ONE 7 (2012) 1–7, https://doi.org/10.1371/journal.pone.0029452.

[44] N. Rahimifard, S. Shoeibi, M.P. Hamedani, M. Saleh, S. Saadati, F. Bagheri, Z. Noori, The microbial quality of poultry paste as raw material, cooked and raw meats in Iran, J. Pure Appl. Microbiol. 3 (2009) 91–93.

[45] A. Abdel-Daim, N. Hassouna, M. Hafez, M.S.A. Ashor, M.M. Aboulwafa, Antagonistic activity of lactobacillus isolates against salmonella typhi in vitro, Biomed Res. Int. 2013 (2013), https://doi.org/10.1155/2013/680605.

[46] J.K. Das, D. Mishra, P. Ray, P. Tripathy, T.K. Beuria, N. Singh, M. Suar, In vitro evaluation of anti-infective activity of a Lactobacillus plantarum strain against salmonella enterica serovar enteritidis, Gut Pathog 5 (2013) 1–11, https://doi.org/10.1186/1757-4749-5-11.

[47] D. Fayol-Messaoudi, C.N. Berger, M.H. Coconnier-Polter, V. Liévin-Le Moal, A. L. Servin, pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against Salmonella enterica serovar typhimurium, Appl. Environ. Microbiol. 71 (2005) 6008–6013, https://doi.org/10.1128/AEM.71.10.6008-6013.2005.

[48] C. Prabhurajeshwar, R. K.Chandrakanth, Development of in vitro methodologies for inhibition of pathogenic bacteria by potential probiotic Lactobacillus sps ; An evidence for production of antimicrobial substances, Int J Pharm Pharm Sci 8 (2016) 10–19, https://doi.org/10.22159/ijpps.2016v8i12.14894.

[49] k.p. rao, g. chennappa, u. suraj, h. nagaraja, a.p.c. raj, m.y. sreenivasa, probiotic potential of lactobacillus strains isolated from sorghum-based traditional fermented food, Probiotics Antimicrob. Proteins. (2015) pp. 146–156. 10.1007/s12602-015-9186-6.

[50] N.C. Gómez, Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of listeria monocytogenes, salmonella typhimurium, and escherichia coli O157 : H7 biofilms formation, Front. Microbiol. 7 (2016) 1–15, https://doi.org/10.3389/fmicb.2016.00863.

[51] J. Woo, J. Ahn, Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens, Lett. Appl. Microbiol. (2013). 10.1111/lam.12051.

[52] Z. Liu, Z. Zhang, L. Qiu, F. Zhang, X. Xu, H. Wei, X. Tao, Characterization and bioactivities of the exopolysaccharide from a probiotic strain of lactobacillus plantarum WLPL04, J. Dairy Sci. 100 (2017) 6895–6905, https://doi.org/10.3168/jds.2016-11944.

[53] J. Magnusson, K. Ström, S. Roos, J. Sjögren, J. Schnürer, Broad and complex antifungal activity among environmental isolates of lactic acid bacteria, FEMS Microbiol. Lett. 219 (2003) 129–135, https://doi.org/10.1016/S0378-1097(02)01207-7.

[54] A.L. Servin, Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens, FEMS Microbiol. Rev. 28 (2004) 405–440, https://doi.org/10.1016/j.femsre.2004.01.003.

[55] Jin Lizhi, Studies On the Mechanisms and Utilization of Probiotics (direct-fed microbials) in Broilers, University Putra Malaysia, Kuala Lumpur, Malaysia, 1996, 1996.