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Methodology

Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic Lactobacilli strains isolated from commercial yoghurt

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ABSTRACT

Milk and milk products have been used by human population since ancient ages and have been a well known source of *Lactobacilli*. The beneficial effects of viable probiotic bacteria as dietary supplements have gained huge research interest, *Lactobacillus* spp. with probiotic characteristics are widely used to prepare fermented dairy products such as yoghurts, milk-shakes etc. The goal of the present study was to examine *Lactobacillus* species with potential activities, total four different companies of Yoghurt samples were collected from City market of Gulbarga region for isolation of probiotic *Lactobacillus* species. Among the samples, 32 lactic acid bacteria strains were isolated, thirteen (13/32) best *Lactobacillus* isolates were selected by preliminary screening as potential probiotics with antimicrobial activity against pathogenic bacteria. All the *Lactobacillus* isolates were then characterized *in vitro* for their probiotic characteristics and antimicrobial activities against pathogens. The isolates were resistant to NaCl (1–6%), bile salt (0.5–3%) and showed good growth in the acidic condition, while maximum growth was observed at pH around 6.0. All the isolates were susceptible to clinical antibiotics; also the isolates were exhibited effective aggregation and hydrophobicity studies. Based on the results, selected *Lactobacillus* isolates were considered as novel and potential probiotic bacteria. Thus, further extensive research on isolation and characterization of probiotic bacteria from local dairy products and their growth optimization might be necessity for development of probiotic enriched food

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supplement and human health benefits through prevention and controlling of bacterial infections.

1. Introduction

The relationship between foods and health benefits is being examined for many years. In recent years, probiotics live and non-pathogenic microorganisms that have beneficial effects on their host's health have attracted wide attention due to growing commercial interest. Research has helped in characterize the specific probiotic organisms and their health benefits [32].

Probiotics are generally used in fermented food production and are considered safe with application in medical and veterinary activities. In the food industry, probiotics are commonly used as starter cultures and have been indexed as part of human microbiota. Yogurt, cheese and fermented milk products are the main sources of probiotics. Lactic acid produced on fermentation of lactose contributes to the sour taste of yogurt by decreasing its pH and enables the formation of the characteristic texture by acting on milk proteins [15]. Traditional dairy products have been used for ages by natives and are the main source of potentially probiotic bacteria.

Despite modern technologies and safety concepts, such as hazard analysis and critical control point (HACCP) system, food-borne illnesses and intoxications are on the rise. According to the Council for Agricultural Science and Technology, microbial pathogens in food cause an estimated 6.5–33 million cases of human illness and up to 9000 deaths annually, the main foods implicated being meat, poultry, eggs, seafood and dairy products. The bacterial pathogens that account for most of these cases include *Salmonella*, *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* [5].

Probiotics are living, health-promoting microorganisms that are incorporated into various kinds of foods. A number of human studies have clearly demonstrated that yogurt containing viable bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*) improves lactose digestion and eliminates symptoms of lactose intolerance. The probiotic bacteria, a component of “thermophillic” starter cultures, used in commercial products today, are mainly members of the genera *Lactobacillus* and *Bifidobacterium* [13]. Also, the ability of probiotics to withstand the normal acidic conditions of gastric juices and bactericidal properties of bile salts, as well as the production of lactic acid that inhibits the growth of other microorganisms, allows them to establish themselves in the intestinal tract [21]. Probiotics produce a wide range of antimicrobial metabolites, i.e., organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins. These activities contribute to microbiological safety by controlling the growth of other microorganisms, and inhibition of pathogenic bacteria [16,23].

Lactobacilli represent a significant part of our intestinal microflora and their role in the general state of human health is being seriously investigated [14]. The genus *Lactobacillus* is one of the major groups of lactic acid bacteria used in food fermentation and is thus of great economical importance. Strains of L. *acidophilus* or closely related species, *Lactobacillus casei*, *L. paracasei* subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* are being increasingly used in novel yogurts [24].

The taxonomy of *Lactobacilli* group has undergone significant changes in recent years causing confusion [26]. A number of studies have been conducted for the identification and classification of LAB including conventional biochemical tests, such as carbohydrate fermentation patterns using commercially available kits, physiological tests [3] as well as more complex molecular-biology methods. More recently, API Kit and Bio-log have been used to a large extent for LAB identification. Several culture media have been developed and evaluated for the selective enumeration of probiotic LAB in yogurts and fermented milk [29,33,35].

In general, *Lactobacilli* have not been associated with disease and have been regarded as non-pathogenic members of intestinal and urogenital flora [1]. *Lactobacilli*, through antagonistic interactions with pathogenic bacteria, maintain the gastrointestinal ecosystem in a healthy state.

Regulatory processes are carried out by species of *Lactobacillus* that produce antibacterial compounds, such as lactic and other organic acids, hydrogen peroxide (H_2O_2) and bacteriocins. Bacteriocins are biologically active, low-molecular weight proteins or peptides that inhibit the growth of a variety of pathogenic bacteria, which appear in bacterial (especially, gastrointestinal) infections.

Therefore, the aim of the present investigation is to identify the potential *Lactobacilli* from phenotypic and genotypic methods, optimize their growth using incubation methods (pour plate and spread plate method) and cultivation conditions like aerobic and anaerobic conditions. The other aim is to determine the in-vitro probiotic characteristics, such as pH, NaCl tolerance, bile, antibiotic susceptibility profile, antimicrobial activity, aggregation studies and cell-surface hydrophobicity capacity of selected probiotic *Lactobacillus* spp. established with pathogenic strains and growth control.

2. Materials and methods

2.1. Samples collection and enrichment

The present study was conducted during December–March 2016 to isolate the strains. Samples of traditional dairy product of different brands (yogurt) were collected considering their popularity among consumers at Gulbarga city and its surroundings. The samples were immediately saved in an icebox until brought to the laboratory for further analysis. The samples were suspended appropriately 10 g of sample in 90 ml of sterile 0.86% normal saline, later enriched and spread on a plate in MRS broth for 18–24 h at 37 °C.

2.2. Preliminary screening and isolation of Lactobacillus spp.

To appropriate isolate of acid and bile-resistant bacteria from the microflora of yoghurt samples, preliminary screening was done for 3–6 h in phosphate buffer solution with pH 3.0 and 6.0. The bacteria *Lactobacillus* spp. were isolated using deMan Rogosa and Sharpe medium. Each sample containing 5–10 g of yogurt was homogenised with sterile phosphate solution (2% w/v) at 30–40 °C in a Stomacher 200–400 circulator (Remi make, India). Later, 1–2 ml of diluted samples were inoculated in MRS broth tube, prepared as per De Man et al., 1960) under aseptic conditions. A control was also run with each batch of samples by pouring distilled water instead of yogurt samples. The inoculated MRS broth tubes were incubated at 37 °C for 24–48 h. MRS broth and agar culture were subjected to different parameters, which affect growth, such as temperature, pH, anaerobic conditions and incubation periods. Tubes were then observed and single colonies were isolated by observing their colony morphology and sub-cultured on petri dishes containing MRS agar and incubated anaerobically for 48 h at 37 °C and the cultures were maintained and stored.

2.3. Morphological and biochemical characterization and identification of Lactobacillus spp.

The isolated bacteria were identified as *Lactobacillus* spp. with the help of various morphological, cultural and biochemical testing techniques as per Bergey's manual. Morphological identification was done by using simple staining, Gram staining and cell morphological tests. Biochemical testing includes catalase, oxidase test, carbohydrate fermentation and milk coagulation. The confirmed *Lactobacillus* isolates were further preserved in MRS broth with skim milk (10%) and glycerol (30%) at −20 °C. At last, the isolated *Lactobacillus* isolates were determined by comparing with some standard *Lactobacillus* species.

3. Characteristics of *Lactobacillus* spp.

3.1. Determination of optimal growth at different pHs

To examine the optimal growth of *Lactobacillus* isolate at different pHs, a single isolated colony was subcultured in MRS broth from 1% (v/v) fresh overnight culture of *Lactobacillus* isolate with pH varying between 2 and 8 and adjusted using NaOH (1.0 M) or HCl (1.0 M) and incubated at 37 °C for 24 h. Afterwards, the growth of bacteria was measured using spectrophotometer, reading optical density at 600 nm against uninoculated broth to observe the ability of the growth of *Lactobacillus* isolates under different pH values [25].

3.2. Bile salt tolerance test

The ability of the strains to endure bile salts was examined as per the modified method of Gilliland and colleagues 1984. This test determines optimal growth after inoculating various isolates individually into MRS broth tubes containing 0.5, 1, 1.5, 2 and 2.5% bile salts. Bacterial growth was observed by measuring absorbance at 600 nm after 18–24 h of incubation at 37 °C. Bile salt-free MRS broth was used as control for this experiment.

3.3. NaCl tolerance test

To conduct NaCl tolerance test, all the isolates were grown on MRS broth at different concentrations of NaCl (1–6%). The broth was inoculated with 10 μl of overnight culture of the isolates and incubated anaerobically at 37 °C for 18–24 h; bacterial growth was observed by measuring absorbance at 600 nm (EFSA, 2008) and NaCl-free MRS broth used as control.

3.4. Molecular identification of Lactobacillus isolate by 16s rRNA

In the present study, 16S rRNA sequencing was used as a tool to identify and confirm the bacterial strains isolated from yogurt. This method is fast and a valid technique for molecular identification. An isolated *Lactobacillus* isolate sequence was commercially sequenced at Genelon Institute of Life Science, Bangalore. Crude sequencing was done by sequencing the BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and homologous hits were studied. Such hit sequences across the species were recovered and examined for multiple alignments using ClustalW on EBI server. Based on scores of multiple alignments, a dendogram was produced and using PHYLIP 3.6 version the evolutionary distance with other similar sequences was generated from different species.

3.5. Antibiotic susceptibility test

Antibacterial susceptibility test is widely performed using disk diffusion method. The method was originally standardised as per ISO 10932/IDF 233 standards with minor modifications [18]. The activated cultures were swabbed on to the agar plates. We have used the antibiotics in the form of dodeca discs (Hi Media, India); these included tetracycline (30 μg), ampicillin (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), cephalotin (30 μg), cotrimoxazole (25 μg), ceftriaxone (30 μg), amoxicillin (10 μg), amoxyclav (10 μg), clindamycin (30 μg), amikacin (10 μg), cefuroxime (30 μg), oxacillin (10 μg), vancomycin (30 μg), Neomycin (10 μg) and sulfamethizole (10 μg) (Hi-Media). The zones of inhibition were noted after incubation at 37 °C for 24 h. Resistance and sensitivity pattern data were interpreted as per the Clinical Laboratory standard institute (CLSI, 2017) [7]. Reference strains of *Lactobacillus fermentum* NCDC 141 *and L. rhamnosus* NCDC 329 were used for quality control for antibiotic susceptibility tests.

4. Antimicrobial activity

4.1. Antagonistic activity of Lactobacillus isolates against pathogens

Antimicrobial activity of all collected *Lactobacillus* isolates against test pathogens was determined by agar-well diffusion method as per Ashraf et al., 2009. *S. aureus* (MTCC 96), *Enterococcus faecalis* (MTCC439), *Klebsiella pneumonia* (MTCC 432), *Pseudomonas aerugenosa* (MTCC 7925), *E. coli* (MTCC 443), *Salmonella typhii* (MTCC734) and *Shigella* spp. (MTCC 13313) were acquired from Medical and Phage Therapy Laboratory, Department of Biotechnology, Gulbarga University, Kalaburagi and used as test pathogens. A 50–100 μl of cell-free supernatant of each *Lactobacillus* isolate was filled in 7 mm diameter well in nutrient agar containing test pathogens. The diameter of the inhibition clear zone was noted after 24 h of incubation. The experiment was performed in triplicate.

4.2. Characterization of antimicrobial substances

The well-selected probiotic *Lactobacillus* isolates were examined for the production of antimicrobial substances like, bacteriocins, organic acids and hydrogen peroxide using agar well-diffusion technique with slight modification of Toure et al., 2003 [34]. The 25-ml grown culture on MRS broth was divided into equal fractions for different assays. For Bacteriocin assay, 5 ml of supernatant was treated with 1 mg/ml pronase or 1 mg/ml trypsin. For organic acids assay, 5 ml of supernatant was adjusted to pH 6.5 ± 0.1 using 1 N NaOH, for hydrogen peroxide assay, 5 ml of supernatant was treated with 0.5 mg/ml of catalase (Hi-Media Pvt Ltd) and treated supernatants were filtered with 0.22 μm pore-size filters (Axiva Pvt Ltd) for bacteriocin assay. A volume of 50–100 μl of each supernatant was placed in 7-mm diameter wells and the plates were swabbed with 1% (v/v) overnight culture of each test pathogen. Inhibitory characteristics were observed and the zone of inhibition was noted after 24 h of incubation at 37 °C.

4.3. Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was studied to assess the phenotypic antimicrobial resistance of a strain to certain probiotic *Lactobacillus* spp. (cell-free culture supernatant). MIC is the lowest *Lactobacillus* spp. concentration that shows no visible growth. The MIC test was conducted using broth-dilution technique as per standard ISO 10932/IDF 233. Serial two-fold dilutions (higher and lower) of CFCS *Lactobacillus* spp. were inoculated with an overnight culture at a final concentration of 10^{7-8} colony-forming unit (cfu/ml). MIC level was determined by measuring the test pathogen's absorbance at 600 nm and *Lactobacillus*-free broth was used as control.

4.4. Autoaggregation of probiotic Lactobacillus *isolates*

Aggregation study was conducted for selected probiotic *Lactobacillus* isolates from yogurt samples on the basis of their deposition properties. Eighteen to 24 h of fresh overnight cultures of each *Lactobacillus* isolates (10^8 cfu/ml) were collected on centrifugation at 6000 × *g* for 20 min, 4 °C, washed twice with phosphate buffer saline (pH 7.2) and the buffer discarded. The autoaggregation percentage was calculated for different *Lactobacillus* isolates after the mixture (vortexed) was incubated at 37 °C for 4 h without agitation.

$$1 - (A_{time}/A_{initial}) \times 100$$

where, A_{time} and $A_{Initial}$ measured at 600 nm represent the absorbance of the mixture at 0 and 4 h.

4.5. Coaggregation of Lactobacillus *isolates with different pathogenic cells*

The coaggregation study examined selected *Lactobacillus* isolates and different test pathogens with slight modifications to Collado et al., 2008 [6]. Bacterial cultures were separately cultured at 37 °C for 24 h in MRS and TSB medium. Bacterial suspension (10^8 cfu/ml) was prepared as described in autoaggregation in the above method, with equal volume of cells of different probiotic *Lactobacillus* spp. and test pathogenic strains (1:1 v/v) mixed and incubated at 37 °C without agitation. Absorbance (A_{600}) of the mixture was studied during incubation at 4 h and the percentage of coaggregation was calculated as,

$$\text{Coaggregation}(\%) = \left[\left(A_{pathogen} + A_{lactobacillus}\right)\Big/2 - A_{mix}\left(A_{pathogen} + A_{lactobacillus}\right)\Big/2 \times 100\right.$$

where, $A_{pathogen}$ and $A_{lactobacillus}$ and A_{mix} represent, respectively, the A_{600} of individual pathogen, *Lactobacillus* spp. and their mixture after incubation for 4 h.

4.6. *Time-kill assay with cell-free culture supernatant (CFCS) of* Lactobacillus *spp. on test pathogens*

Time-kill assay was conducted by co-culturing pathogenic cells and cell-free culture supernatant (CFCS) of *Lactobacillus* spp. and 300 μl of pathogenic suspension (10^8 cfu/ml) was added to 15 ml of CFCS of different *Lactobacillus* isolates. The CFCS was adjusted to pH 6.5 and incubated at 37 °C. At initial and planned intervals, fractions were separated by serially diluting and placing them on LB agar to determine the surviving cells of individual pathogens.

4.7. *Cell-surface hydrophobicity*

Cell-surface hydrophobicity was examined after different *Lactobacillus* spp. and test pathogens partition individually into xylene from PBS [31]. The cells were washed twice with PBS, the optical density (A) at 540 nm was adjusted to 0.5 ± 0.01–1.0 ml of bacterial suspension, 60 μl xylene was added and vortexed for 1 min, and the optical density of the water phase was determined. Percentage of hydrophobicity was calculated as per the formula of Raouf et al., 2013 [29].

$$\left(1 - A_{after} \middle/ A_{before}\right) \times 100$$

4.8. *Scanning electron microscopic study*

As small changes in cell morphology of bacteria are difficult to notice under light microscope, SEM was used to identify changes or any rupture in cell morphology of the populations due to the effect of *Lactobacillus* spp. [29]. The selected pathogens were grown on TSB media with increasing antibiotic concentrations and normal pathogen. The bacterial cells from each culture were recovered by centrifugation at 6000 rpm/min and the cells were washed twice with potassium phosphate buffer (50 mM, pH 7.0). Bacterial cells were then fixed by soaking in 2.5% glutaraldehyde in potassium phosphate buffer (50 mM, pH 7) and incubated overnight at 4 °C. The specimens were later washed twice with buffer and dehydrated in ethanol in a series (v/v) ranging from 30, 40, 50, 60, 70, 80 and 90–100% and stored in 100% ethanol. For SEM, the specimens were dried to critical point, coated with gold and inspected with an S-200C. Results were correlated with standard pathogenic culture and control selected pathogens.

4.9. *Quantification of organic acid and determination of pH value*

One percent (v/v) 24 h active culture of effective *Lactobacillus* isolates was used to inoculate 10% sterilized skim milk existed from Hi-Media Pvt Ltd, Bangalore, and the initial pH of 6.76 was set on a digital electrode pH meter. The inoculated skim milk was incubated at 37 °C for 72 h, samples assembled every 12, 24, 48 and 72 h and liquids of coagulated milk were separated by filtration. The pH of the separated liquid was recorded using a digital electrode pH meter and the organic acid was quantified through titration with 0.1 N NaOH.

5. Results

5.1. *Isolation and identification of* Lactobacillus *isolates*

Four different brands of yogurt samples were collected from local milk vendors. *Lactobacillus* spp. were screened and isolated from 13 (40.62%) of the total 32 bacterial strains; *Lactobacillus* spp. were predominantly isolated (Table 1).

After culturing for 24–48 h, 13 strains were selected (as forming wide and white) colonies on the selected MRS agar plate (Fig. 1); they were later identified as *Lactobacillus* spp. by observing their colony morphology, physiological as well as biochemical characterization (Table 2). All the results clearly show that bacteria were Gram-positive, rod shaped (Fig. 2), non-motile and catalase negative.

Table 1
Origin and Number of isolates from yoghurt sample after screening for *Lactobacillus* spp.

Source of dairy product (yoghurt)	No. of isolates	No. of *Lactobacillus* spp.
Nilgiris sweetened natural	10	6
Epigamia (greek yoghurt)	7	3
Milky mist Flavoured yoghurt	6	1
Milma partially skimmed yoghurt	9	3
Total	32	13 (40.62%)

The confirmed *Lactobacillus* isolates were named Y1, Y2, Y3, Y4, Y5, Y6, Y7, Y8, Y9, Y10, Y11, Y12 and Y13. These isolates were cultured on MRS with glycerol (30%) broth and stored at −20 °C.

5.2. *Characterization of* Lactobacillus *isolates*

5.2.1. *Determination of optimal growth at different pHs*

All the isolated *Lactobacillus* spp. from different yogurt samples have shown maximum growth; the *Lactobacilli* isolated from Nilgiris sweetened yogurt and Epigamia (Greek Yoghurt) were observed at pH 6–7.0. The O.D. reading is the average value of the two samples (OD = 2.982) (Fig. 3).

5.2.2. *Tolerance to bile salt and NaCl*

The isolated *Lactobacillus* spp. were capable of growing and surviving in 0.05–2.5% bile salt; at this concentration, all the *Lactobacillus* isolates multiplied timely (Fig. 4), (optical density values against different bile salt concentration of each *Lactobacillus* isolates). Also, the *Lactobacilli* from yogurt samples tolerated 1–6% NaCl (Fig. 5). The isolates of *Lactobacillus* have resistance to different concentrations of bile salt and NaCl and are capable of being probiotic.

5.3. *Molecular identification of* Lactobacillus *isolate*

The *Lactobacillus* strain ckLfm was identified by 16srRNA sequencing, after initial analysis at NCBI and RDP II (http//:rdp.cme.msu.edu); appropriate sequences were downloaded and phylogenetic

Fig. 1. Single screened colonies on MRS media.

Table 2
Morphological, cultural and biochemical characteristics of isolated *Lactobacillus* spp. from yoghurt samples.

Sl. no	Selected *Lactobacillus* spp.	Morphological and cultural Characteristics	Gram's staining	Motility test	Catalase test	Carbohydrate fermentation test			
						Glucose		Sorbitol	Sucrose
						Acid	Gas		
1	Y1	1mm, White, shiny smooth, round	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	+ve	+ve
2	Y2	Small, 0.1–0.5mm, circular and round	Gram +ve, bacilli	Non motile	Negative	−ve	−ve	+ve	+ve
3	Y3	1mm, White, shiny smooth, round	Gram +ve, bacilli	Non motile	Negative	−ve	−ve	+ve	+ve
4	Y4	1mm, White, shiny smooth, round Shiny,	Gram +ve, bacilli	Non motile	Negative	−ve	−ve	+ve	+ve
5	Y5	1.0 mm white, rough, irregular and round	Gram +ve, bacilli	Non motile	Negative	−ve	−ve	−ve	−ve
6	Y6	Small Circular, colourless	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	−ve	−ve
7	Y7	Small, 0.1–0.5mm, rough dull and round	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	+ve	+ve
8	Y8	1.0 mm white, rough, irregular and round	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	+ve	+ve
9	Y9	Small Circular, white creamy	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	−ve	−ve
10	Y10	Small, 0.1–0.5mm, rough dull and round	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	−ve	+ve
11	Y11	Small Circular, white creamy	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	+ve	+ve
12	Y12	1.0 mm white, rough, irregular and round	Gram +ve, bacilli	Non motile	Negative	+ve	−ve	−ve	+ve
13	Y13	Small, 0.1–0.5mm, rough dull and round	Gram +ve, bacilli	Non motile	Negative	−ve	−ve	−ve	+ve

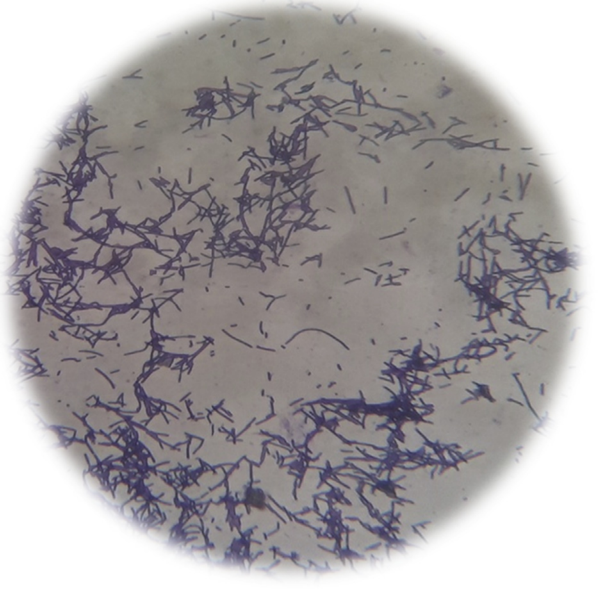

Fig. 2. Microscopic observation of Gram's stained *Lactobacillus* spp.

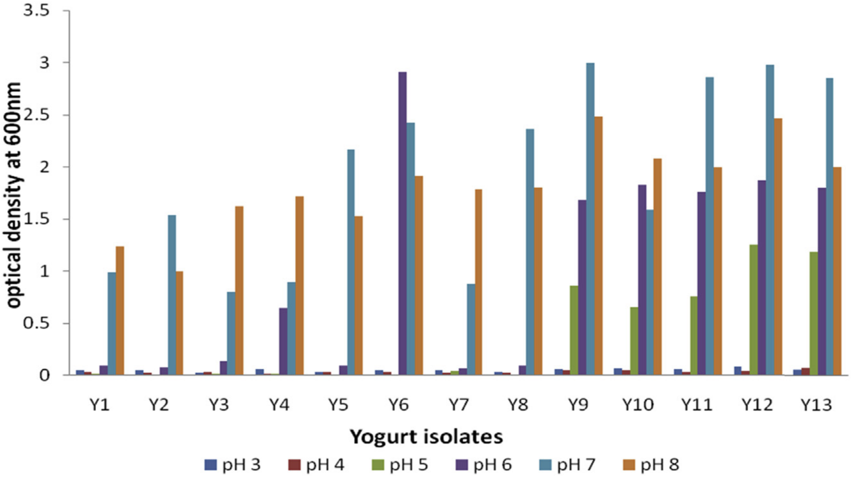

Fig. 3. Optimal growth and pH of isolated *Lactobacillus* isolates from yoghurt samples.

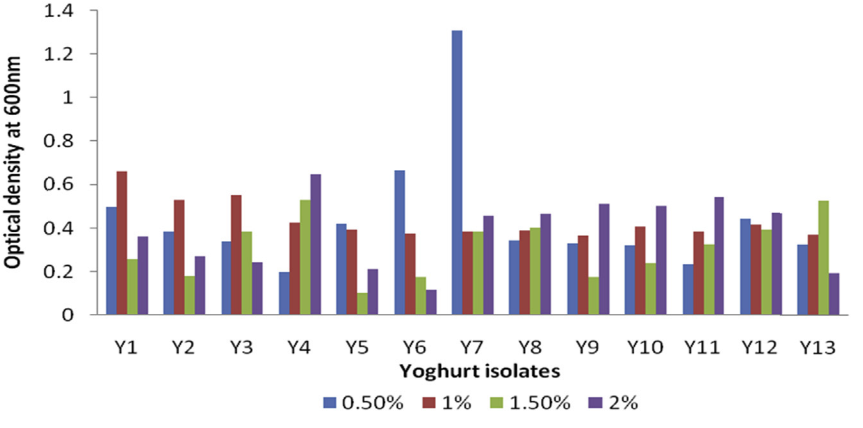

Fig. 4. Bile acid tolerance of *Lactobacillus* isolates from yoghurt samples.

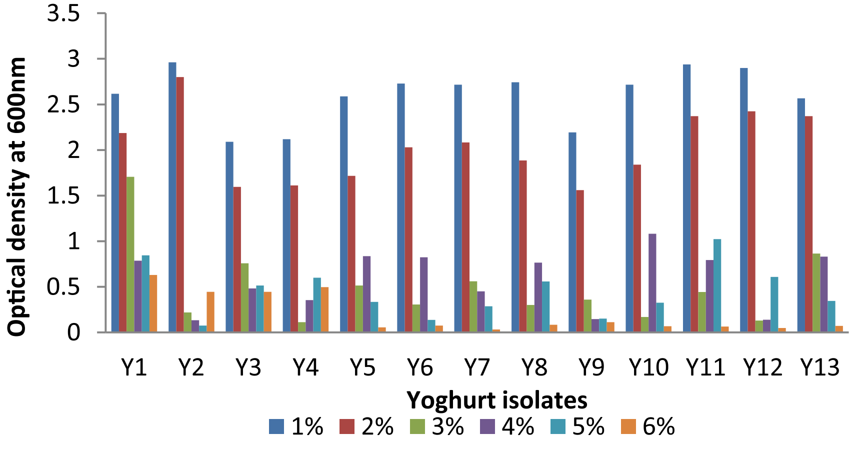

Fig. 5. NaCl tolerance of *Lactobacillus* isolates from yoghurt samples.

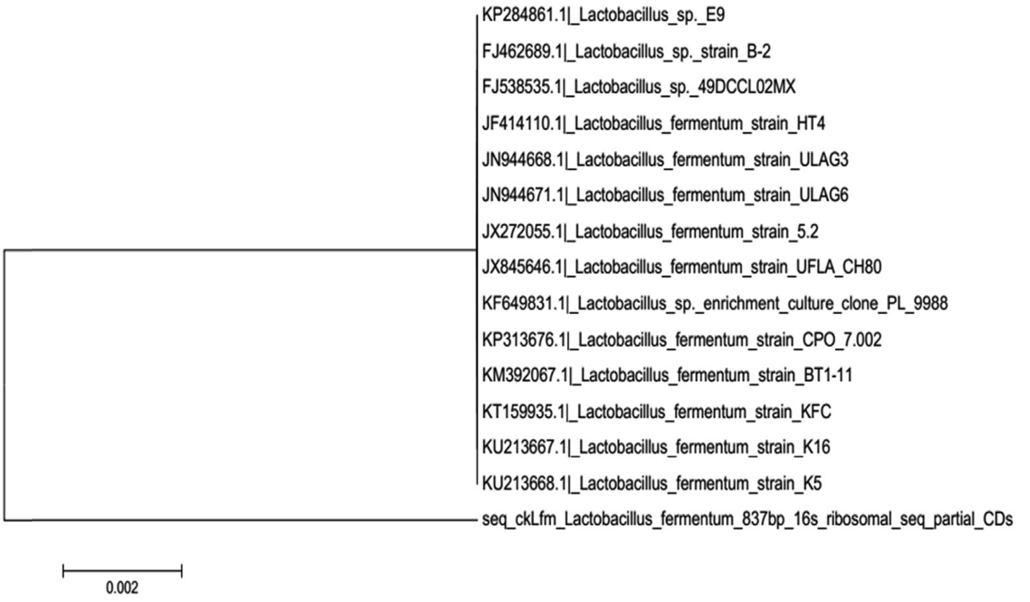

Fig. 6. Phylogenetic tree based on neighbour-joining method of 16S rRNA gene sequencing of *Lactobacillus* isolate.

analysis was performed and a tree was constructed using ClustalW software, which showed the phylogenetic relationship of the isolates (Fig. 6). The analysis of the sequence showed 99% similarity with the sequence reported on *Lactobacillus* spp. and consequently isolates of ckLfm were confirmed as *Lactobacillus fermentum*. The 16srRNA isolate sequence was deposited in the GenBank, and was assigned the accession number KX242349.

5.4. Antibiotic susceptibility test

An antibiotic susceptibility test was carried out on 13 positive potential probiotic *Lactobacillus* spp. from yoghurt samples against 15 antibiotics from different groups. Isolates were sensitive to amoxyclav, cephalothin, co-triamaxazole, erythromycin, tetracycline, sulfamethizole and amikacin, some displayed intermediate resistance to cefuroxime, clindamycin, gentamycin, vancomycin, chloramphenicaol and ciprofloxacin, and prominent resistance was to ampicillin and oxacillin. Among the 13 *Lactobacilli* isolates, 10 (76.92%) were resistant to ampicillin, 10 (76.92%) were sensitive to cefuroxime and 11 (7.69%) had shown intermediate resistance to chloromphenicol (Table 3 and Fig. 7).

6. Antimicrobial activity

6.1. *Antagonistic activity of* Lactobacillus *isolates against pathogens*

Selected *Lactobacillus* isolates were examined for their antimicrobial activity using modified agar-well diffusion method. The antagonistic effects of *Lactobacillus* isolates were exposed to indicator microorganisms, such as *S. aureus, E. faecalis, K. pneumonia, Pseudomonas aerogenosa, E. coli, S. typhii* and native isolated *Shigella* spp. All 13 *Lactobacillus* strains and reference strain showed antagonistic effects against all indicator microorganisms tested, but the degree of antagonism varied among the *lactobacillus* strains. All the isolated *Lactobacillus* strains exhibited average inhibition (15–25 mm) on the growth of test pathogens, but the Y9, Y10 and Y13 isolates were the most effective ones in inhibiting the test pathogens (19–33 mm). Overall, many of the isolated *Lactobacillus* strains showed better antagonistic activity against the test pathogen than the reference strain *Lactobacillus fermentum*

Table 3
Antibiotic susceptibility test for yoghurt samples.

Sl. no.	Antibiotics Used	No. of resistance	No. of sensitive	No. of intermediate
1	Ampicillin	10 (76.92%)	–	3 (23.07%)
2	Amikacin	5 (38.46%)	8 (61.53%)	–
3	Amoxyclav	6 (46.15%)	7 (53.84%)	–
4	Carbenicillin	5 (38.46%)	7 (53.84%)	1 (7.69%)
5	Cefuroxime	2 (15.38%)	10 (76.92%)	1 (7.69%)
6	Chloramphenicol	4 (30.76%)	8 (61.53%)	11 (7.69%)
7	Ciprofloxacin	6 (46.15%)	7 (53.84%)	–
8	Cotrimoxazole	6 (46.15%)	7 (53.84%)	–
9	Gentamycin	5 (38.46%)	8 (61.53%)	–
10	Kanamycin	5 (38.46%)	8 (61.53%)	–
11	Neomycin	5 (38.46%)	8 (61.53%)	–
12	Streptomycin	9 (69.23%)	4 (30.76%)	–
13	Sulfamethizole	4 (30.76%)	9 (69.23%)	–
14	Tetracycline	4 (30.76%)	9 (69.23%)	–
15	Vancomycin	4 (30.76%)	9 (69.23%)	–

(Table 4 and Figs. 8 and 9). To ascertain if the isolates are bacteriostatic or bacteriocidal, a confirmation test was done by modified agar overlay method; in this case, swabs were taken from each clear zone of the test organism and were streaked on to nutrient agar plates for growth. Depending on growth, the bacteriostatic and bacteriocidal activities were classified (Table 5). The growth of the indicator organism was interpreted as an inhibitory activity, called bacteriostatic, while no growth was interpreted as bacteriocidal.

6.2. *Characterization of antimicrobial substances*

Effective *Lactobacillus* isolates from yogurt samples were considered for the characterization of inhibitory substances like Bacteriocin, organic acid and hydrogen peroxide. The antimicrobial substance produced by the three *Lactobacillus* isolates was characterized by agar well-diffusion assay

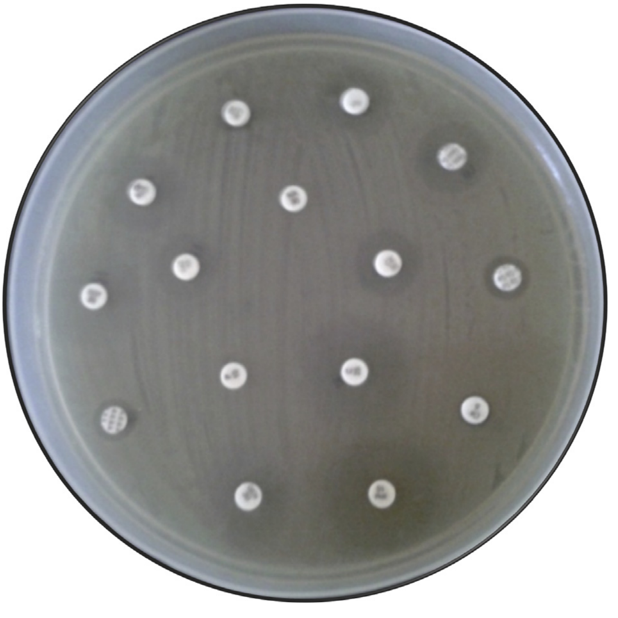

Fig. 7. Antibiotic susceptibility pattern of *Lactobacillus* isolates.

Table 4
Antagonistic activity of *Lactobacillus* isolates against test pathogens from yoghurt samples.

Sl.No	*Lactobacillus* isolates	Zone of Inhibition in mm (from outer edge of *Lactobacillus* colony to outer edge of clear zone)						
		S. aureus	*S. typhi*	*E. coli*	*E. faecalis*	*K. pneumoniae*	*P. aerogenosa*	*Shigella sps*
1	Y1	21	19	18	22	14	18	19
2	Y2	24	24	24	35	19	20	24
3	Y3	19	16	22	26	18	18	12
4	Y4	19	26	20	18	23	20	24
5	Y5	17	14	24	19	19	19	14
6	Y6	22	19	20	21	17	19	23
7	Y7	19	20	18	18	20	15	13
8	Y8	16	23	17	13	21	17	19
9	Y9	18	22	17	19	15	18	22
10	Y10	31	30	28	34	29	27	27
11	Y11	10	12	10	12	18	11	20
12	Y12	22	14	20	19	18	13	21
13	Y13	29	19	24	33	31	22	16

Whereas Y – yoghurt isolate.

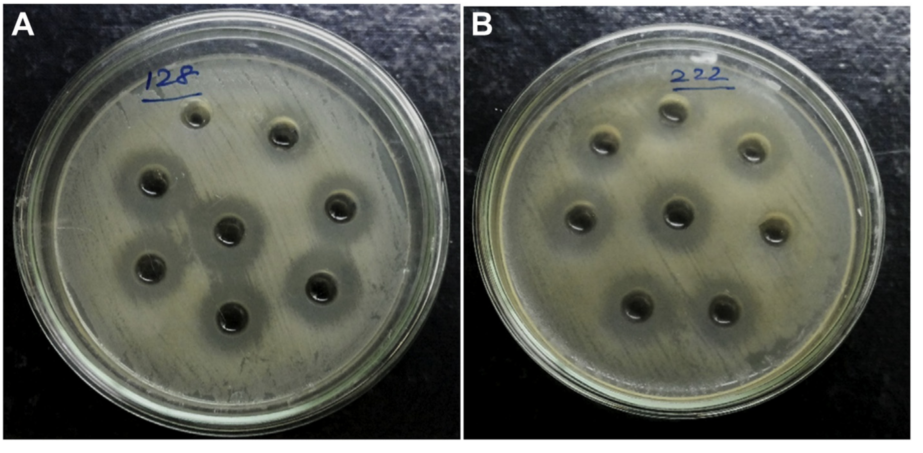

Fig. 8. Antagonistic activity of *Lactobacillus* isolates against test pathogens. A. Gram-positive, B. Gram-negative.

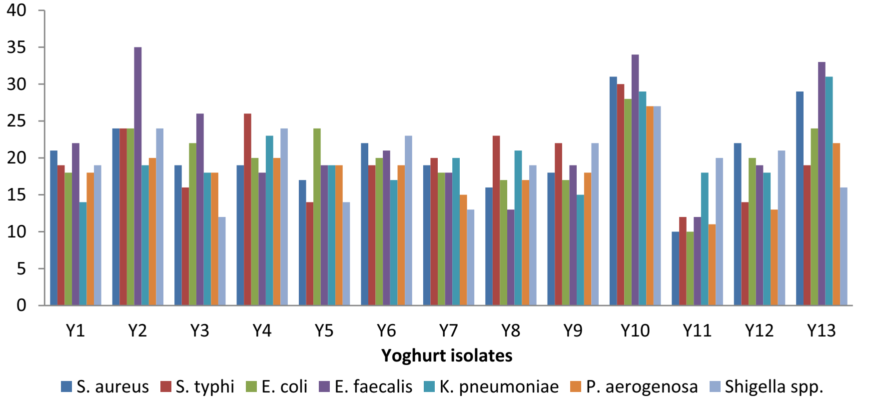

Fig. 9. Antagonistic activity of *Lactobacillus* isolates from yoghurt samples against test pathogens.

Table 5
Bacteriostatic and bactericidal activity of yoghurt isolates.

Name of the pathogens	Y9 isolate	Y10 isolate	Y13 isolate
S. aureus	–	–	+
E. faecalis	+	+	–
E. coli	–	–	–
P. aerogenosa	–	+	+
K. pneumoniae	+	–	+
S. typhii	+	+	–
Shigella spp.	–	–	–

+ = bacteriostatic, – = bacteriocidal.

Table 6
Characterization of antimicrobial substances of selected yoghurt isolate.

Sl no.	*Shigella* selected strains	Y9 (in mm)		Y10 (in mm)		Y13 (in mm)	
		Bacteriocin assay	Organic acid assay	Bacteriocin assay	Organic acid assay	Bacteriocin assay	Organic acid assay
1	*S. aureus*	22	–	–	17	–	12
2	*E. faecalis*	–	16	–	11	–	10
3	*E. coli*	21	–	–	18	–	17
4	*P. aerogenosa*	–	22	–	13	–	16
5	*K. pneumoniae*	–	–	–	10	–	14
6	*S. typhii*	17	18	–	11	–	18
7.	*Shigella* spp.	18	16	–	15	–	19

against different indicator strains. The results showed that culture supernatants of all the three isolated *Lactobacillus* isolates (Y9, Y10 and Y13) and the reference strain treated with pronase (1 mg/ml) or trypsin (1 mg/ml) did not affect their inhibitory activities against the indicator strains. This indicates that the inhibitory effect of *Lactobacillus* isolates was not due to bacteriocin production. Culture supernatants treated with catalase also did not affect the inhibitory activities of the *Lactobacillus* isolates against indicator strains. This showed that inhibition by the *Lactobacillus* isolates was not due to hydrogen peroxide production. However, neutralized supernatant (pH 6.5) of all three *Lactobacilli* did not have any inhibitory effect on the *Lactobacillus* isolates but due to their organic acid production (Table 6 and Fig. 10). Hence, this study concludes that of the three *Lactobacillus* isolates, Y10 and Y13 isolates were bacteriocin and Y9 was responsible for both organic acid and bacteriocin production.

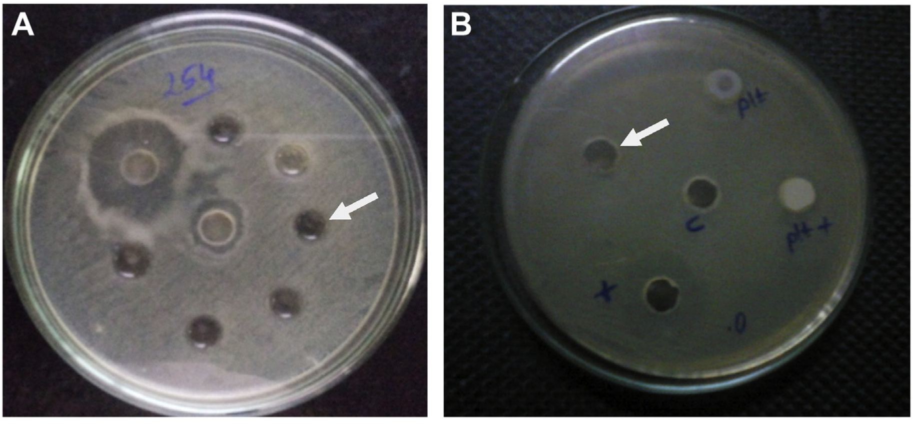

Fig. 10. Antimicrobial activity of *Lactobacillus* (CFCS) inhibitory substances against test pathogen. A. Bacteriocin, B. Organic acid.

6.3. *Minimal inhibitory concentration of cell-free culture supernatant (CFCS) of* Lactobacillus *isolates*

The selected CFCS of *Lactobacillus* isolates were used for MIC assay. The results show that MIC for Y9 isolate was 50 μl against *S. aureus, k. pneumoniae, E. coli and p. aerogenosa*, 100 μl against *E. faecalis, S. typhii* and *Shigella* spp; Y10 isolate showed MIC of 100 μl against *E. faecalis, S. typhii, S. aureus, K. pneumoniae, E. coli* and 120 μl against *P. aerogenosa* and *S. higella* spp. and Y13 isolate of 100 μl against *P. aerogenosa, Shigella* spp. *E. faecalis, S. typhii, S. aureus, K. pneumoniae* and 128 μl against *E. coli.*

6.4. *Auto and coaggregation of probiotic* Lactobacillus *isolates*

The autoaggregation study examined all the three *Lactobacillus* isolates and different test pathogens based on their deposition ability. The results revealed that among the three, Y13 isolate had the highest percentage of autoaggregation after 24 h of incubation (52%) as compared to Y9 and Y10 isolates (Table 7a).

The coaggregation results of the *Lactobacillus* isolates tested with different test pathogens are shown in Table 7b. This study is strain-specific as compared to aggregation; among the isolates, Y9, Y10 and Y13 showed effective coaggregation with *Shigella* sps at 18.9, 17.6 and 19.6%, respectively; similarly, Y10 isolate showed less coaggregation ability with *S. aureus*, and also with other test pathogens used.

6.5. *Time-kill assay with CFCS of* Lactobacillus *isolates on test pathogens*

Time-kill assay illustrates the reduction in cell count of different test pathogens in the presence of CFCS of each of the *Lactobacillus* isolates (Y9, Y10 and Y13) covering 2–3 fractions of different incubation periods (6, 12, 18 and 24 h). The inhibition activity was clear in the case of Y13 as compared with Y9 and Y10. The study concludes that inhibitory substances like bacteriocin and organic acids present in the CFCS of isolates are responsible for killing of pathogens (Fig. 11).

6.6. *Cell-surface hydrophobicity*

Cell-surface hydrophobicity studies the potential association between physico-chemical features and its efficient adherence to the intestinal mucus. The results show that it varies with test pathogens used in the selected *Lactobacillus* isolates; Y13 isolate (54%) had the highest hydrophobic nature, compared to the other isolates, which had lesser or no hydrophobicity towards xylene from the control considered 0%. Among the test pathogens, *Shigella* spp and *E. coli* (31 and 26.2%) had better hydrophobicity percentage, *P. aerogenosa* 17.5% and *S. typhii* 13.2%, but *K. pneumonia* (7.6%), *S. aureus* (4.9%) and *E. faecalis* (5%) had lower percentage of hydrophobicity (Table 8).

Table 7a
Percentage of autoaggregation of probiotic selected isolates with pathogenic strains.

	Auto-aggregation (%)		
	4 h	18 h	24 h
***Lactobacillus* strains**			
Y9 isolate	19 ± 2.3	27 ± 3.4	45 ± 2.8
Y10 isolate	13 ± 2.5	20 ± 2.5	39 ± 2.7
Y13 isolate	22 ± 1.1	28 ± 1.2	52 ± 1.7
Pathogenic strains			
Staphylococcus aureus	2.7 ± 1.0	3.6 ± 0.1	5.1 ± 0.9
Enterococcus faecalis	2.1 ± 1.4	2.7 ± 0.4	3.5 ± 0.8
E. coli	7.1 ± 1.2	12.3 ± 0.8	15.9 ± 1.2
Pseudomonas aeruginosa	3.6 ± 0.8	11.4 ± 1.1	21 ± 1.5
Klebsiella pneumoniae	5.4 ± 1.1	13 ± 1.3	15 ± 1.1
Salmonella typhi	2.9 ± 0.8	10.2 ± 0.9	19.5 ± 0.1
Shigella spp.	2.6 ± 0.4	9.4 ± 0.1	21 ± 1.0

All data are expressed in mean ± SD, n = 6, where as Y – yoghurt isolate.

Table 7b
Percentage of coaggregation of *Lactobacillus* isolates with test pathogens.

Selected Lactobacillus strains	% of co-aggregation with indicator strains						
	S. aureus	*E. faecalis*	*E. coli*	*P. aerugenosa*	*K. pneumoniae*	*S. typhii*	*Shigella* spp.
Y9 isolate	17.2 ± 2.12	11.2 ± 0.6	17.5 ± 0.4	13 ± 0.7	12 ± 2.1	16 ± 0.65	18.9 ± 5.3
Y10 isolate	2.4 ± 3.20	9.5 ± 0.36	6.6 ± 1.12	2.5 ± 0.61	7 ± 0.52	7 ± 0.43	17.6 ± 4.6
Y13 isolate	12.6 ± 1.51	15.8 ± 0.52	14.4 ± 0.51	8.4 ± 0.81	10 ± 1.12	12 ± 0.31	19.6 ± 1.4

All data are expressed in mean ± SD, n = 6, Where as Y – yoghurt isolate.

6.7. *Scanning electron microscopic study*

The upshots of cell morphology of test pathogen treated with cell-free culture supernatant of *Lactobacillus* species were inspected by SEM and any alteration or rupture in the cell morphology was observed as shown in Fig. 12.

6.8. *Quantification of organic acid and determination of pH value*

Isolates from yogurt samples (Y9, Y10 and Y13) coagulated the skim milk and organic acids produced were detected by titrimetric method (Table 9).

7. Discussion

The aim of this work was to isolate and characterize potential probiotic bacteria from yogurt samples of Gulbarga and its surrounding region to assess their anti-bacterial activity against some

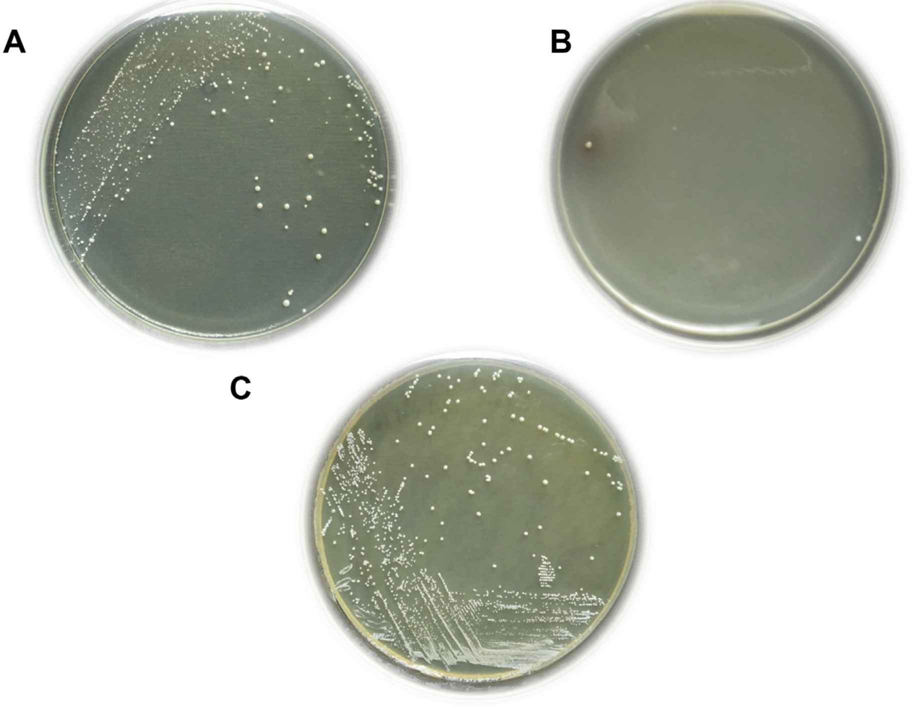

Fig. 11. Time kill assay of *Lactobacillus* (CFCS) isolate against test pathogen. A. Reduction in pathogen colonies, B. Complete killing of pathogen colonies, C. Control.

Table 8
Percentage of cell-surface hydrophobicity of bacterial strains.

Selected strains	Cell-surface hydrophobicity (%)
Y9 isolate	32.0 ± 0.11
Y10 isolate	26 ± 0.52
Y13 isolate	54 ± 1.0
Test pathogens	
Staphylococcus aureus	4.9 ± 0.1
Enterococcus faecalis	5 ± 0.2
E. coli	26.2 ± 0.1
Pseudomonas aeruginosa	17.5 ± 1.6
Klebsiella pneumoniae	7.6 ± 0.1
Salmonella typhi	13.2 ± 0.1
Shigella spp.	31.0 ± 0.1

All data are expressed in mean ± SD, n = 6, where as Y – yoghurt isolate.

common pathogenic bacteria. Based on morphological characteristics, 13 isolates were identified as *Lactobacillus* spp. from yoghurt samples. After Gram-staining the isolated bacteria were rod-shaped, convex, rough, smooth, shiny, irregular, circular, Gram-positive, facultative anaerobic, nonspore forming, which indicate them to be the member of *Lactobacillus* spp. [19]. The effective growth of the isolates at pH 6.5 on MRS-agar plates in anaerobic conditions further confirmed their identification as *Lactobacillus* spp. [9]. Oxidase, catalase and IMViC test of selected isolates gave the same results as *Lactobacillus* spp. All the isolates were indole, MR, VP, citrate, oxidase and catalase negative, and the results are similar to the findings of Elizete and Carlos, 2005. Carbohydrates used in this study, all the 13 isolates were able to ferment glucose, sorbitol and sucrose suggesting that they are capable of growing in a variety of habitats consuming a range of carbohydrates.

pH can dramatically affect bacterial growth. To be used as probiotic, organisms have to tolerate low pH of human gut. The pH in the human stomach ranges from 1.5 to 4.5 depending on the intervals of feeding, the types of food consumed and the duration of food digestion, which can take up to 3–4 h. As the results in Fig. 3 reveal, all the collected *Lactobacillus* spp. showed high tolerance and maximum isolates were tolerant to the range of pHs and grow well in acidic pH. Bile salts also constitute an important factor for considering the viability of *Lactobacillus* spp. [4]. In the present study, 0.5–2% bile salts were supplemented in the growth media, as it corresponded to that available in the human intestinal tract, and 0.3% is the maximum concentration among healthy men [13]. Therefore, before selection of probiotic bacteria for human consumption it must endure 0.3% bile concentration [28]. *Lactobacillus* spp. isolated in this study was resistant to 0.3–0.6% bile salt. All the isolates survive and grow in 0.6% bile salt concentration (Fig. 4). NaCl is an inhibitory substance, which may inhibit the growth of certain types of bacteria, and probiotic organisms have to withstand high salt concentration

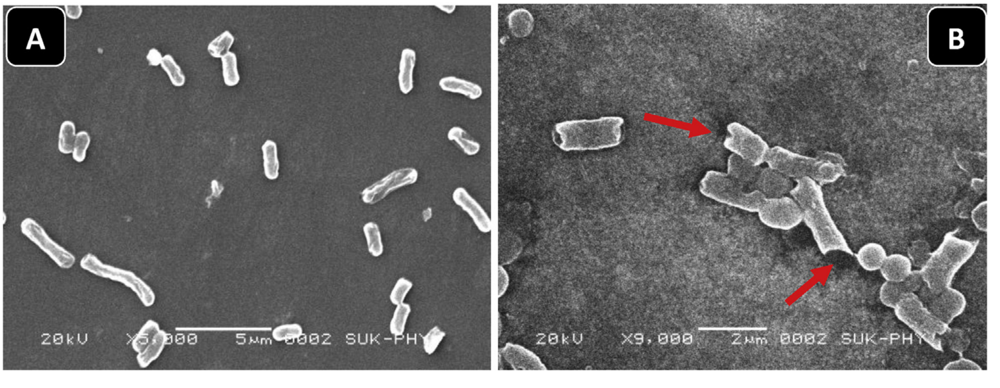

Fig. 12. Scanning Electron Microscopy images. [A] Control, [B] Pathogen treated with *Lactobacillus* isolate (CFCS).

Table 9
Quantification of organic acid and determination of pH value of selected *Lactobacillus* spp.

Sources of bacteria	Name of the bacteria	Incubation time (Hour)	Incubation temp. (°C)	Organic acid (%)	pH
Yoghurt	Y9	12	37	0.31	5.71
		24	37	0.35	5.01
		48	37	0.38	4.61
		72	37	0.37	4.51
	Y10	12	37	0.12	5.46
		24	37	0.24	5.12
		48	37	0.26	4.72
		72	37	0.35	4.50
	Y13	12	37	0.12	5.62
		24	37	0.26	5.21
		48	37	0.29	4.70
		72	37	0.32	4.41

in human gut. The present results showed that *Lactobacillus* spp. isolated from yogurt was able to tolerate 1–9% of NaCl and good growth was observed at 1–5% NaCl (Fig. 5).

The present assessment reveals that organic-acid production increased with incubation time and the pH of the media decreased with increasing acid production. From the results, the highest acidity (0.3 ± 0.2) and the lowest pH (4.45 ± 0.2) were observed after 72 h of incubation at 37 °C for *Lactobacillus* spp. from all the samples. This investigation shows a slight variation in organic acid production by *Lactobacilli* due to regional variation (Table 9). Antimicrobial activity is one of the most important selection patterns for effective and novel probiotics. The isolates acquire antimicrobial effects by producing some substances, such as organic acids (lactic, acetic, propionic acids, succinic acid, etc.), hydrogen peroxide, low-molecular weight antimicrobial substances and bacteriocins [11]. Probiotics together with *Lactobacillus*, *Bifidobacterium* and *Streptococcus* sps are known to be inhibitory to the growth of a wide range of intestinal pathogens in humans. In addition to positive effects against disease caused by an imbalance in the gut microflora, several experimental observations have revealed a potential protective effect of probiotic bacteria against the development of colon tumours [14].

In the study of Osuntoki et al., 2009, *Lactobacillus* spp. isolated from fermented dairy products exhibited antibacterial activity against some clinically important pathogens, such as Enterotoxigenic *E. coli* (4.2 mm), *Salmonella typhimurium* (4.3 mm) and *L. monocytogenes* (5.0 mm). Isolates of the present study have superior antimicrobial ability than the *Lactobacillus* spp. isolates. Our isolates showed almost similar antagonistic activity against *E. coli* and *S. typhimurium* in contrast to *Lactobacillus plantarum* and *Lactobacillus salivarius* isolated by Murray et al., 2004 [21] from a plant-related probiotic. Gharaei-Fathabad and Eslamifar 2001 studied a strain of *Lactobacillus paraplantarum* isolated from tea leaves demonstrating strong inhibitory activity against *S. typhii* (65 mm), *E. coli* (30 mm), *S. aureus* (56 mm), *E. faecalis* (55 mm) and *Citrobacter sps* (60 mm). Isolates of the present study have virtually similar antimicrobial potential. In our study, antagonistic activity of selected *Lactobacillus* isolates against seven different test pathogens showed noticeable activity (Figs. 8, 9 and Table 4) and reach that level with organic acid and low-molecular weight antimicrobial substances produced from the isolates (Fig. 10).

Aggregation between microorganisms of the same strain (auto-aggregation) or between genetically different strains (co-aggregation) is of great consequence in several ecological niches. Aggregating bacteria may reach an adequate mass to form biofilms or adhere to the mucosal surfaces of the host and thus develop their functions [8]. In the current study, the autoaggregation assay was examined for three effective selected *Lactobacillus* strains and the bacterial pathogens on the basis of their deposition characteristics. The results of this study show that autoaggregation prolonged as a concern of time and was the highest after 4 h of incubation (Tables 7a and b).

8. Conclusion

Lactobacillus strains isolated in this study from the yogurt samples commercially available in Gulbarga market with *in vitro* features make them potential candidates for probiotic application. The strains of the isolates showed attractive probiotic properties, such as excellent pH, NaCl and bile tolerance, aggregations, suppression of pathogen growth under *in vitro* conditions. Moreover, all tested strains were susceptible to a number of clinically effective antibiotics. These results suggest that isolates from yogurt samples have potential properties essential for novel probiotics. More research is needed to exploit other potential probiotic properties as the *Lactobacillus* species showed more efficient properties as probiotic than earlier reports suggest. Also, this study provides support for the formulation of novel probiotics as a biotherapeutic agents or supplements for prevention of bacterial gastro-intestinal infection and other related enteric infections. Further, *in-vivo* trials are needed to establish whether they function as probiotics in real-life situations for human health benefit.

Ethical approval

This Medical Biotechnology and Phage Therapy Laboratory, Department of Biotechnology approved ethical clearance by Institutional Clearance Certificate (IECC) for *in-vitro* and *in-vivo* studies.

Authors Contribution

First author is responsible for carrying out the research work, data analysis and optimization of experimental work and Corresponding author is responsible for research planning executing and providing valuable inputs and in writing manuscript.

Conflict of interest

We declare that no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yclnex.2018.10.001.

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