International Food Research Journal 27(1): 131 - 140 (February 2020)
Journal homepage: *http://www.ifrj.upm.edu.my*

Dairy origin lactobacilli: functional analyses and antagonistic potential against multidrug-resistant foodborne pathogens

[1]Kabir, S., [2]Shahid, M., [1]Waseem, M., [1]Muzammil, S., [1]Nawaz, Z., [1]Rasool, M. H., [3]Shahzad, A., [1,4]Hayat, S., [1]Taj, Z., [1]Khurshid, M., [5]Rizvi, N. B., [1]Waheed, S., [1]Nisar, M. A. and [1]*Saqalein, M.

[1]*Department of Microbiology, Government College University Faisalabad, Pakistan*
[2]*Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Pakistan*
[3]*Department of Clinical Sciences, Gomal University, Dera Ismail Khan, Pakistan*
[4]*Department of Biotechnology, University of Sargodha, Pakistan*
[5]*Institute of Chemistry, University of the Punjab, Lahore, Pakistan*

Article history

Received: 6 September 2019
Received in revised form: 22 December 2019
Accepted: 7 January 2020

Keywords

lactobacillus, probiotics, cell-free culture supernatant, multidrug-resistant pathogens

Abstract

The present work was designed to characterise novel indigenous strains of lactobacilli and to identify and evaluate their antagonistic activity against enteric pathogens. A total of four lactobacilli strains were isolated from Dahi samples (continental yogurt) and characterised using phenotypic and biochemical tests, and by 16S rDNA sequencing. The isolates were identified as *Lactobacillus paracasei* SABA3, *L. paracasei* SABA4, *L. fermentum* SABA5, and *L. casei* SABA6. The probiotic potential of these strains was assessed under the gastrointestinal simulating environment. The antagonistic activity of lactic acid bacilli (LAB) strains against multidrug-resistant (MDR) test pathogens was determined by *in vitro* assays. Moreover, the bactericidal activity of cell-free culture supernatant (CFCS) was determined by agar well diffusion and growth inhibition assays. All the LAB strains were tolerant to 0.3% bile salts, extreme acidic conditions (stomach environment) as well as pH > 6.5 (small intestine); and can efficiently grow at 37°C. Moreover, the isolates were found as non-haemolytic which confirms their safety and use as potential probiotics. The genetic screening has shown the presence of *qnr*S, *tet*K, *tet*W, *van*R, *van*X, and *qnr*A antibiotic-resistant determinants. The *L. fermentum* SABA5 exhibited the strongest bactericidal activity against the pathogenic bacterial species among all of these potential probiotic strains. The CFCS inhibited the proliferation of pathogenic bacterial species in a concentration-dependent manner. The chromatographic profiling of all isolates depicted lactic acid as a major fermentation product.

Introduction

Gastrointestinal tract (GIT) infections are increasingly being common and are often caused by bacteria, viruses, or parasites, and characterised by vomiting, diarrhoea, and many other symptoms (Tam *et al.,* 2012). Bacterial pathogens are found responsible for approximately 20 - 40% of diarrheal cases but are associated with a higher number of deaths in children particularly in developing countries like Pakistan; and substantial financial loss in the developed parts of the world. The epidemiological patterns are changing therefore the number of *Salmonella* infections is declining in industrial economies with more diarrheal cases associated with *Escherichia coli* infections (Fhogartaigh and Dance, 2013). The indiscriminate use of antimicrobial agents for the treatment of GIT as well as other infections has led to the emergence of antibiotic-resistant phenotypes among the bacterial species. An alternate approach is to use beneficial bacteria for the management of GIT infections. Probiotics are the living microorganisms that impart health benefits to host when ingested in sufficient number (Khan *et al.,* 2013; Khurshid *et al.,* 2015; Shahid *et al.,* 2017). Generally, processed fermented foods contain probiotics that confer beneficial effects particularly in various GIT diseases. Therefore, these microorganisms have the potential to replace or support the traditional therapies against enteropathogens. Among probiotics, lactobacilli are a more versatile and valuable group of bacteria with reference to the production of various volatile organic acids, antibacterial peptides, and intrinsic resistance to commonly used antimicrobial agents (Saadatzadeh *et al.,* 2013).

The human gut microbiota vary among different ethnic populations, age groups, geographical regions and dietary habits including the traditional

*Corresponding author.
Email: drsaqalein@gcuf.edu.pk

fermented foods (Afolayan *et al.,* 2019). Thus far, no indigenous probiotic strain is commercially available in Pakistan, and the market is having probiotic products with western strains. Therefore, it is a challenge for local researchers to explore indigenous probiotic strains whose specific health claims and safety are scientifically proven and clinically validated in the local settings. Taking these problems into consideration, and to find a safe and efficacious alternative; the present work was designed to identify and evaluate the antagonistic activity of novel indigenous strains of lactobacilli against pathogenic *Salmonella* and *Escherichia coli*.

Materials and methods

Bacterial isolates and culture conditions

Four different lactobacilli were isolated from Dahi (continental yogurt) and cultivated in MRS (DeMan-Rogosa-Sharpe, pH 6.5, HiMedia® Laboratories, India) medium supplemented with 0.05% L-cysteine (Sigma Aldrich®, USA). The medium was incubated at 37°C for 24 h under a strict microaerophilic environment unless otherwise mentioned. The pathogenic bacteria *Salmonella* Typhi and *Escherichia coli*, isolated from clinical samples were cultivated on Luria-Bertani (LB) medium (Sigma Aldrich®, USA) at 37°C for 24 h under aerobic conditions.

Biochemical profiling of isolates

For the assessment of metabolic diversity of lactobacilli strains, various sugar fermentation (adonitol, arabinose, dulcitol, glucose, lactose, and sorbitol) and biochemical tests (indole, Simmon's citrate, oxidase, urease, lysine and ornithine decarboxylation, acetoin, gas, and H_2S production/biosynthesis) were performed as per Bergey's Manual (Vos *et al.,* 2011); whereas, biochemical profiling of pathogenic microorganisms was performed using API® 20E kit (bioMérieux™, France).

Molecular typing of bacterial strains

The FavorPrep™ Tissue DNA Extraction Mini Kit (Favorgen® Biotech Corporation, Taiwan) used to extract the total genomic DNA and 16S rDNA was amplified by commercially available degenerate primers (27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT) followed by sequencing from Macrogen™ (South Korea) (Bouchet *et al.,* 2008). The obtained sequences were analysed *in silico* and submitted to GenBank to obtain their accession number. The evolutionary status of isolates was determined using the online program Phylogeny.fr (http://www.phylogeny.fr/) and the phylogenetic tree was constructed.

Antibiotic resistance profiling and pathogenicity testing of enteropathogens

Congo red binding assay was performed to evaluate the pathogenicity of *E. coli.* Briefly, the bacterium was cultured on Congo Red agar and incubated at 37°C overnight, followed by an additional incubation at 25°C for 48 h. *E. coli* strain which developed red colour colonies between 18 and 72 h of incubation was considered as CR positive (Priti *et al.,* 2016). Kirby-Bauer disc diffusion assay was performed to study the resistance pattern of pathogenic *Salmonella* and *E. coli*. Different antibiotics (Oxoid™, UK) including amikacin (30 µg), amoxicillin (30 µg), ampicillin (10 µg), aztreonam (30 µg), cefotaxime (30 µg), cephradine (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), ofloxacin (5 µg), tetracycline (30 µg), and vancomycin (30 µg) were used and results were interpreted as per CLSI recommendations (CLSI, 2015). Moreover, the modified double-disc synergy test (DDST) was performed for the detection of ESBL (Extended Spectrum β-Lactamases) producing *E. coli* (Kaur *et al.,* 2013).

Assessment of probiotic potential of lactobacilli

The probiotic potential of lactobacilli was assessed by monitoring growth patterns under different physiochemical conditions as below:

Acid tolerance

The ability of the LAB isolates to thrive under acidic conditions was studied by cultivation in MRS broth (HiMedia® Laboratories, India) at a wide-ranging pH spectrum i.e. 1.5 to 8.0, and bacterial density was recorded as an indicator of bacterial growth.

Bile tolerance

The bile salt resistance of LABs was monitored by cultivation in MRS agar (HiMedia® Laboratories, India) supplemented with 0.3% (w/v) of sodium deoxycholate (Sigma Aldrich®, USA). Tolerance to bile salts was measured by the appearance of colonies on agar plates.

Thermal stability and growth curve

LABs were cultivated in MRS broth (HiMedia® Laboratories, India) and kept under incubation at a temperature ranging from 22 to 42°C to measure the optimum temperature. Afterward, the bacterial cell density was recorded as an indicator of bacterial

growth. Finally, the growth curves of lactobacilli were plotted by recording absorbance at 630 nm wavelength.

Safety assessment of lactobacilli

The haemolytic activity and antibiotic resistance profiling were selected for assessment of the safety of isolated lactobacilli as below:

Haemolytic activity

LABs were cultured on Columbia agar (Oxoid™, UK) plates supplemented with 5% defibrinised sheep blood, and allowed to grow under standard cultural conditions. On the very next day, plates were observed for complete haemolysis (β-haemolysis), partial haemolysis (α-haemolysis), and no haemolysis (γ-haemolysis) (Pieniz *et al.,* 2014).

Antibiogram analysis

The antibiogram of isolates was studied by disc diffusion (Kirby-Bauer method) and broth microdilution assays (Cheesbrough, 2006).

Kirby-Bauer disc diffusion assay

The antimicrobial susceptibility of probiotics was tested using 11 different antibiotic discs (Oxoid ™, UK) namely; amikacin (30 µg), amoxicillin (30 µg), ampicillin (10 µg), aztreonam (30 µg), cefotaxime (30 µg), cephradine (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), ofloxacin (5 µg), tetracycline (30 µg), and vancomycin (30 µg) were used. The susceptibility/resistance was interpreted as per CLSI guidelines (CLSI, 2015).

Broth micro-dilution assay

The broth microdilution method was used to measure the MIC (Minimum Inhibitory Concentration) of each antimicrobial agent. Different concentrations of antibiotics used in study were as follows: amikacin (2 - 256 µg/mL), amoxicillin (2 - 32 µg/mL), ampicillin (0.015 - 2 µg/mL), ceftriaxone (0.125 - 512 µg/mL), ceftazidime (2 - 128 µg/mL), ciprofloxacin (0.05 - 64 µg/mL), imipenem (0.125 - 128 µg/mL), kanamycin (16 - 128 µg/mL), tazobactam (2 - 256 µg/mL), and vancomycin (2 - 512 µg/mL). Briefly, the suspension of bacterial isolates equivalent to 0.5 McFarland standard and appropriate concentration of antibiotics were inoculated in 96-well microplate, followed by incubation under standard cultural conditions. Optical density at 630 nm was regularly recorded after an interval of 1 h.

Genetic screening of antibiotic resistance genes

Probiotic strains exhibiting resistant phenotypes were subjected to the genetic screening of antibiotic resistance determinants. Various primer sets (Table 1) were used for detection of 11 different antibiotic resistance genes i.e., *vanR, vanX, mefA, mefE, tetK, tetW, blaZ, qnrA, qnrB1, qnrB2,* and *qnrS* (Liu *et al.,* 2009).

Table 1. Specific primers for the amplification of various antibiotic resistant determinants.

Resistance Trait	Gene	Primer Sequence	Reference
Vancomycin resistance	*vanR*	AGCGATAAAATACTTATTGTGGA CGGATTATCAATGGTGTCGTT	(Klein *et al*., 2000)
	vanX	TCGCGGTAGTCCCACCATTCGTT AAATCATCGTTGACCTGCGTTAT	(Liu *et al*., 2009)
Macrolides	*mefA*	CTATGACAGCCTCAATGCG ACCGATTCTATCAGCAAAG	(Liu *et al*., 2009)
	mefE	ATGGAAAAATACAACAATTGGAAACGA TTATTTTAAATCTAATTTTCTAACCTC	(Liu *et al*., 2009)
Tetracycline resistance	*tetK*	TTATGGTGGTTGTAGCTAGAAA AAAGGGTTAGAAACTCTTGAAA	(Gevers *et al*., 2003)
	tetW	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	(Aminov *et al*., 2001)
β - lactam resistance	*blaZ*	TACTTCAACACCTGCTGCTTTCG CATTACACTCTTGGCGGTTTCAC	(Liu *et al*., 2009)

Evaluation of antagonistic activity against gastro-intestinal tract pathogens

Antibacterial potential of isolates and cell-free culture supernatant (CFCS) was assessed against selected GIT pathogens namely multi-drug resistant (MDR) *E. coli* and *S.* Typhi by multiple assays as below:

Cross streak line assay

LABs were inoculated in the centre of Tryptic Soy (Oxoid™, UK) and Nutrient agar (Oxoid™, UK) plates and incubated under appropriate cultural conditions followed by two-hour chloroform gas inactivation. Then, the test pathogens were plated perpendicular to the central streak line of LABs in duplicate and further incubated at 37°C. Antibacterial activity was measured by the zones of inhibition around the central streak line of LABs (Annuk *et al.,* 2003).

Co-culture assay

The cell suspensions of lactobacilli and test pathogens (0.5 McFarland) were co-cultured in Brain-Heart Infusion (BHI) broth (Oxoid™, UK), whereas the monoculture of test pathogens was taken as control. BHI co-cultures were serially diluted and cultivated on MacConkey agar (Oxoid™, UK) and CFU/mL was calculated. Mathematically, % inhibition was estimated using Eq. 1 (Lim and Im, 2009):

$$\% \text{ Inhibition} = [(\text{CFU/mL in control} - \text{CFU/mL in co-culture}) / \text{CFU/mL in control}] \times 100 \quad \text{(Eq. 1)}$$

Agar well diffusion assay

The suspension of the test organisms (0.5 McFarland) was inoculated on BHI agar; then 6 mm diameter wells were punched into the agar and into each well LAB CFCS was inoculated; whereas the sterile MRS broth was added in the control well. The size of the inhibition zones was estimated in mm.

Microplate growth inhibition assay

In a microtiter plate, the test pathogen *S.* Typhi and *E. coli* (previously cultured in BHI and LB broth, respectively) were mixed with appropriate CFCS dilutions (5, 10, and 15% v/v) and incubated under standard conditions. The optical density at 630 nm was recorded as a function of bacterial growth for 8 h. Finally, graphs were plotted between bacterial growth (absorbance) and time (h) (Lash *et al.,* 2002; Saadatzadeh *et al.,* 2013).

Biochemical characterisation of CFCS

Initially, the effect of pH, temperature, and proteinase K on CFCS was monitored to assess the stability of CFCS (Mariam *et al.,* 2014). Later, TLC (Thin Layer Chromatography) was performed to decipher the organic acids profile of CFCS. Briefly, the silica gel coated TLC plate (stationary phase) and a mixture of acetone, ammonium hydroxide, ethanol, chloroform, water (60:22:10:6:2) mobile phase was used. The solution of 10% (w/v) acetic acid, ascorbic acid, citric acid, formic acid, butyric acid, propionic acid, and lactic acid were used as standards. The 0.25 g methyl red and bromophenol blue (indicator solutions) dissolved in 100 mL of 70% methanol was used for plate development (Lee *et al.,* 2001).

Results

Molecular typing of dairy lactobacilli strains

A total of four phenotypically different Gram-positive, non-spore former and non-motile rods were screened and labelled as L3, L4, L5, and L6. All isolates were unable to metabolise citrate, urea, phenylalanine, lysine, and ornithine. Their ability to ferment a range of carbohydrate depicts their metabolic diversity.

The sequence analysis of lactobacilli 16S rDNA of L3 revealed the highest homology (identity 99%) with *L. paracasei* (Accession No: JX254904, isolation source Korean kimchi) and L4 displayed maximum homology with *L. paracasei* (Accession No: JQ247981, isolation source Korean makgeolli). However, the L5 strain depicted the highest levels of homology with *L. fermentum* (Accession No: AB680190, isolated from Japan) and L6 isolate showed maximum homology with *L. casei* (Accession No: JN974882, isolation source Korean cow's raw milk). The L3, L4, L5, and L6 were submitted to GenBank as *L. paracasei* SABA3 (Accession No: KX599355), *L. paracasei* SABA4 (Accession No: KX599356), *L. fermentum* SABA5 (Accession No: KX599357), and *L. casei* SABA6 (Accession No: KX599358), respectively (Figure 1).

Molecular identification and antibiogram analysis of test pathogens

Based on API® 20E (bioMérieux, France) identification system and 16rDNA sequence analysis, test pathogens were identified as *Escherichia coli strain* SABA3 (Accession No. KY305421) and *Salmonella enterica* subsp. *enterica* serovar Typhi SABA10 (Accession No. KY305432). Additionally, antibiograms were also determined for pathogens, and *E. coli* strain SABA3 was found ESBL positive and resistant to penicillin, ampicillin, cefuroxime,

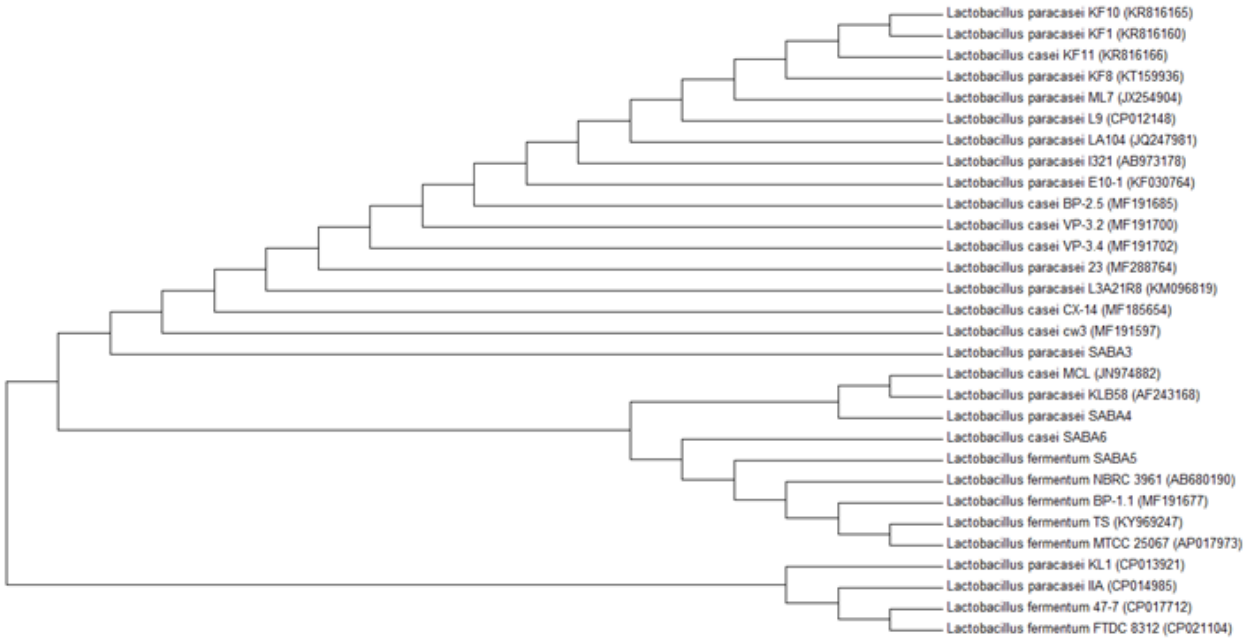

Figure 1. Phylogenetic tree generated by Neighbour Joining method; representing the cladistic position of *L. paracasei* SABA3, *L. paracasei* SABA4, *L. fermentum* SABA5, and *L. casei* SABA6.

cefixime, cefotaxime, ceftazidime, ceftriaxone, moxifloxacin, ciprofloxacin, and was sensitive to amikacin, meropenem, sulbactam/cefoperazone, and tazobactam/piperacillin. *S.* Typhi strain SABA10 was found resistant to penicillin, ampicillin, cefuroxime, cefixime, cefotaxime, ceftazidime, ceftriaxone, sulfamethoxazole/trimethoprim, tetracycline, and was only sensitive to amikacin, tazobactam/piperacillin, and meropenem.

Probiotic potential of lactobacilli strains

All isolates were able to tolerate bile salt and acid stress displaying their capability to thrive in the harsh environment of the stomach. *L. paracasei* SABA4 and *L. casei* SABA6 yielded maximum growth at pH 6.0, whereas *L. paracasei* SABA3 and *L. fermentum* SABA5 displayed optimal growth at pH 5.5 and 5.0, respectively. Surprisingly, *L. paracasei* SABA3 showed efficient growth at pH 2.5, representing its ability to survive in the stringent environment of the stomach (Figure 2C). The *L. paracasei* SABA3 and *L. casei* SABA6 showed optimum growth at 25°C, whereas, the *L. paracasei* SABA4 and *L. fermentum* SABA5 displayed maximal growth at 37°C (Figure 2B).

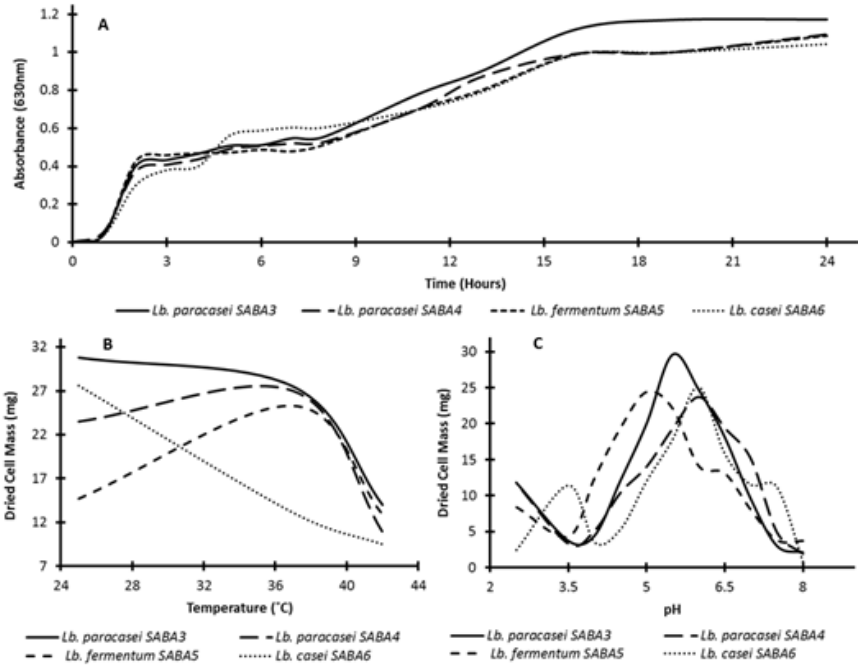

Figure 2. Growth curves of isolated lactobacilli at 37°C (A). All isolates could tolerate broad range of temperature (B) and pH range (C).

Safety assessment of lactobacillus strains

The haemolytic activity and antibiogram profiling were scrutinised for safety assessment. All isolates were found as non-haemolytic (γ-haemolysis). According to Kirby-Bauer disc diffusion assay, all isolates were resistant to ampicillin, amoxicillin, vancomycin, ciprofloxacin, amikacin, gentamicin, cefotaxime, cephradine, aztreonam, and tetracycline. However, *L. casei* SABA6 was susceptible to ofloxacin. Briefly, the antibiogram of *L. paracasei* SABA3 and *L. paracasei* SABA4 displayed insensitivity against ampicillin, while the rest of the drugs disturbed the log phase. However, tazobactam and ampicillin did not affect the growth of *L. fermentum* SABA5 and *L. casei* SABA6 while all other tested antibiotics prolonged lag phase.

Antibiotic resistance genes

The disc diffusion assay has shown that the LAB isolates displayed resistance to vancomycin, tetracycline, quinolones, and β-lactams. The genes *bla*Z, *mef*A, *mef*E, *qnr*B1, and *tet*M were absent; whereas, *qnr*S, *tet*K, *tet*W, *van*R, and *van*X genes were found in all isolates. The *qnr*A was found in *L. paracasei* SABA4 and *L. fermentum* SABA5 only.

Antagonistic activity of lactobacillus strains against GIT pathogens

The antagonistic potential of isolates was assessed against MDR *E. coli* and *S.* Typhi by a variety of *in vitro* growth inhibition assays. All isolates have shown considerable inhibitory activity against pathogens as observed in the streak line method. Furthermore, lactobacilli-pathogen co-culture assays demonstrated strong growth inhibitory activity of lactobacilli against test pathogens. All isolates reduced the growth of ESBL producing MDR *E. coli* up to 90%, whereas nearly 50% growth of MDR *S.* Typhi was reduced. Based on the streak line and co-culture assay, *L. fermentum* SABA5 harboured strong antibacterial activity against test GIT pathogens. Later, the antagonistic activity of cell-free culture supernatant (CFCS) was also recorded by agar well diffusion and microplate growth inhibition assays. The results of CFCS were comparable to the streak line assay. However, the microplate inhibition assay demonstrated that CFCS hindered bacterial growth in a concentration-dependent manner; nearly 10% CFCS was enough to halt *E. coli* growth whereas at least 15% CFCS was required for partial inhibition of *S.* Typhi growth (Figure 3).

Stability and biochemical nature of CFCS

CFCS of all isolates retained their antibacterial activity against test pathogens even after proteinase K and pH treatments, while the inhibitory effect of CFCS was lost following heat treatment. CFCS of *L. fermentum* SABA5 and *L. casei* SABA6 were relatively more resistant to pH and proteinase K treatment. The TLC has shown that all isolates synthesised lactic acid as a major fermentation product. Stability assays and TLC results indicate the presence of volatile organic acid(s) and certain antibacterial peptides responsible for bactericidal activities.

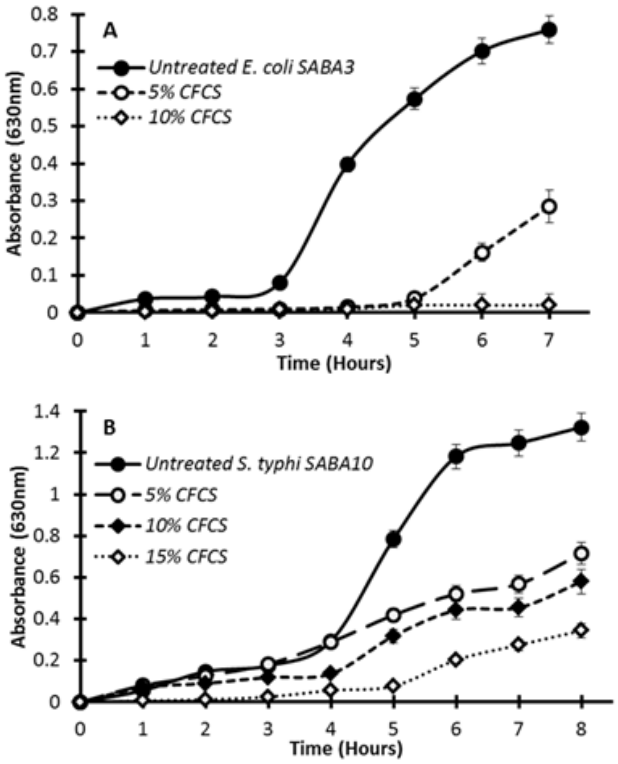

Figure 3. CFCS (cell-free culture supernatant) can effectively suppress the proliferation of ESBL producing MDR *E. coli* (A) and MDR *S.* Typhi (B).

Discussion

The present work suggested that LABs can prevent GIT infections due to their ability to synthesise a number of substances that possess antibacterial properties such as bacteriocins and organic acids, immunomodulation mechanisms in the intestine of the host (Rowland *et al.,* 2010), and competitive exclusion of enteropathogens (Lee and Puong, 2002). The increasing antibiotic resistance among the pathogens has compelled the researchers to discover alternatives to classical antibiotic therapy. The use of probiotics is one of these alternatives to counter the resistance bacterial pathogens (Singh *et al.,* 2011).

The present work was carried out to examine the antibacterial activity of LABs against pathogenic

MDR *E. coli* and S. Typhi. A total of four strains of *L. paracasei* SABA3, *L. paracasei* SABA4, *L. fermentum* SABA5, and *L. casei* SABA6 from Dahi (continental yogurt) were isolated. The abundance of *Lactobacillus* strains in the fermented milk product represents their capability to resist the acidic environment. Like other LABs, all isolates were found to utilise a wide range of sugars as carbon source, which represents the metabolic and fermentative diversity of isolates, and their ability to produce various organic acids (Vos *et al.,* 2011). The probiotic potential of putative LAB strains was assessed in the conditions simulating the GIT. Further, all of the LABs strains survived at 0.3% bile salts concentration and at pH 2.5 (Figure 2C). The resistance to acid environment is mainly attributed to the presence of potent transcription regulated mechanism monitoring the function of H^+-ATPase pumps, modifications in the cell envelope, production of chaperones, and shock-related proteins (Cotter and Hill, 2003). Resistance to extreme acidic pH and bile salt stress confers survival benefits in the harsh environment of the mammalian stomach. However, probiotics must first colonise the human intestine to provide maximum benefits to their host. In the intestine, the environment is different from the stomach. The survival of LAB isolates at pH > 6.5 displays their ability to survive in the upper intestinal tract as well (Huang and Adams, 2004). While tolerance to bile salts is attributed to the bile salt hydrolase system, the isolates must harbour such a system to thrive the bile stress (Jayashree *et al.,* 2013). It was found in the present work that the optimum temperature for growth was 37°C for *L. paracasei* SABA4 and *L. fermentum* SABA5, while *L. paracasei* SABA3 and *L. casei* SABA6 depicted optimum growth at 25°C (Figure 2B). Studies have reported that different strains have different optimum temperatures for growth that usually range between 20 and 45°C, although growth at 10°C and as high as 53°C has also been recorded depending on the lactobacilli species (Yang *et al.,* 2018).

The haemolytic activity and antibiotic resistance were examined for the safety concerns among the LABs, and all the strains were found as non-haemolytic as shown in previous studies (Hawaz, 2014). The LAB strains were resistant to nearly all antibiotics tested in the present work. The results could be due to the fact that the probiotic bacteria are inherently resistant to many antibiotics and this resistance is chromosomally encoded and non-transferrable. The results correlate with previous reports (Balamurugan *et al.,* 2014; Singh *et al.,* 2015). Importantly, all isolates lack *bla*Z, *mef*A, *mef*E, *qnr*B1, and *tet*M genes, and harboured *qnr*S, *tet*K, *tet*W, *van*R, and *van*X antibiotic resistance determinants. The vancomycin resistance is attributed to a gene cluster (consisting of seven different genes) situated on the large-sized conjugative plasmid (Liu *et al.,* 2009). The *mef* genes (efflux pumps) provide resistance against macrolides and none of the isolates were found to harbour the *mef* gene. The *tetK* gene is responsible for the efflux system, while *tetW* is a ribosomal protection system against tetracycline (chelating drugs) (Chopra and Roberts, 2001). The quinolone resistance among our isolates is attributed to the *qnr* genes namely *qnr*S and *qnr*A.

Strong bactericidal and antagonistic properties of LABs are attributed to the production of various antimicrobial agents and metabolites including antimicrobial peptides, peroxides, small volatile organic acids, ethanol, and various other potent organic molecules (Šušković *et al.,* 2010). Furthermore, the phenomenon of competitive exclusion, the competition between LABs and the pathogenic bacteria for food from the host and attachment sites plays a significant role in improving the health of the mammalian host (Saulnier *et al.,* 2009). Computational genomic analysis of lactobacilli revealed the presence of 18 different types of bacteriocins responsible for bacteriocinogenic activity e.g., *L. fermentum* harbours colicin V (Drissi *et al.,* 2014). In the present work, the antagonistic potential of LAB strains against ESBL producing MDR *E. coli* and MDR *S.* Typhi was evaluated by various *in vitro* assays. Our results are in accordance with previous studies related to *Salmonella* (Hudault *et al.,* 1997; Balamurugan *et al.,* 2014) and *E. coli* (Hutt *et al.,* 2006). The cross-streak method resulted in clear zones of inhibition against test pathogens. These findings are coherent with earlier report in which antagonistic activity using the same assay was demonstrated in a microaerobic and anaerobic environment (Annuk *et al.,* 2003). The antibacterial activity of CFCS was tested by agar well diffusion assay. It was found that the diameter of inhibition zones varied from 12 – 15 mm. Similar results had been described in the published data regarding the antibacterial activity of lactobacilli against the same targeted pathogens (Tejero-Sarinena *et al.,* 2012; Mehanna *et al.,* 2013).

Different antibacterial compounds were present in the cell-free supernatant (CFCS) of lactic acid bacteria which were responsible for their antagonistic effects against pathogens. The inhibitory effect depended upon the concentration of the CFCS and therefore a strong inhibitory activity could be

achieved with increasing concentration of CFCS. We reported that the concentrations of 10 and 15% were found effective to inhibit or suppress the growth of MDR *E. coli* and *S.* Typhi, respectively. In previous reports, the MDR *E. coli* did not grow well at the concentrations (10 and 15%) while the growth of *S.* Typhi was highly suppressed at similar concentrations (Das *et al.,* 2013). The co-culture assay revealed the strong antibacterial activity of LAB by exhibiting significant differences in the number of colonies as compared to the control. The highest percentage of inhibition was observed in the case of *L. fermentum* SABA5 at 95% for *E. coli* and 57% for *S.* Typhi (Figures 3). The nature of antimicrobial compounds present in CFCS was determined by giving a variety of treatments, and we found that CFCS retained its antibacterial activity following proteinase K enzyme and heat treatment but lost their effect when the pH of the supernatant was adjusted to 6.5 - 6.8. The findings indicated that the antibacterial activity in our LAB strains was mainly due to the production of organic acids which lowers the pH of the medium. When the pH of the medium was increased, the antibacterial effect was lost (Bilkova *et al.,* 2011). However, there are reports which indicate that only low pH is not sufficient to target *Salmonella*. Therefore, further studies are required to identify other antimicrobial metabolites produced by the LAB isolates (Hudault *et al.,* 1997). Similar to the previous findings, the analysis of CFCS depicted that the isolates synthesised lactic acid as a major fermentation product. The studies have emphasised the role of lactic acid and other volatile organic acids in the antibacterial activity of lactobacilli (Shokryazdan *et al.,* 2014).

Conclusion

The present work revealed the probiotic potential and antibacterial activity of lactic acid bacteria against the test pathogens. The antibacterial potential reflects a bright chance for probiotics to replace or support the traditional antibiotics. The present work suggested the use of lactic acid bacteria as a starter culture in functional foods and nutraceuticals. However, the future prospects of the present work include the assessment of the efficacy of these lactic acid bacteria to cure and treat different diseases, and their safety by further investigation in animal models along with the evaluation of physicochemical properties of antimicrobial substances produced, host-microbiome interactions using advanced genetic, proteomic, and molecular techniques. Further, the clinical trials involving human subjects are warranted followed by the approval from concerned regulatory authorities that will pave the way for commercialisation of indigenous probiotic strains and help to support the national income.

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