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Lactic acid bacteria from kefir grains: Potential probiotics with antagonistic activity against multidrug resistant Gram-negative bacteria

Maryam Ghane*, Laleh Babaeekhou, Babak Montazeri Najafabadi and Mahara Sadat Mirmostafa

Department of Biology, Islamshahr Branch, Islamic Azad University, Islamshahr, Iran.
Email: ghane@iiau.ac.ir

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ABSTRACT

Aims: This study aimed to isolate and identify lactobacilli strains that have antagonistic activity against multidrug resistant (MDR) isolates of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and evaluate their probiotic properties.
Methodology and results: Twenty-one *Lactobacillus* isolates were collected, and their antimicrobial activity was assessed by agar well diffusion, broth microdilution and time-kill test. The probiotic potential of the isolates was evaluated as well. The bacterial culture and cell free supernatant (CFS) of all isolates exhibited antibacterial activity against all MDR isolates. Out of 21 isolates, 4 isolates (A31, B35, S20 and S25) displayed the highest antimicrobial activity and further evaluated. The minimum inhibitory percentages of CFS from selected isolates against pathogens ranged from 10 to 30% and the bactericidal percentages ranged from 20 to 50%. The inhibitory activity of CFS was not changed after heating but abrogated as the pH neutralized. The growth kinetic of the MDR pathogens was significantly reduced in the presence of the CFS of all isolates. The isolates had a less than 1-log reduction in their viability in acid tolerance test and could grow in the presence of 0.3% bile salts. Strains S20, S25 and B35 exhibited high co-aggregation with *E. coli* (51.7-73.3%), *P. aeruginosa* (53.7-69.3%) and *K. pneumoniae* (49.7-65.3%). Molecular identification revealed that the isolates were *Lactobacillus rhamnosus* (B35) and *Lactobacillus paracasei* (S20, S25, A31).
Conclusion, significance and impact of study: The results suggest that these lactobacilli isolates may have potential applications for controlling and preventing colonization of infections caused by MDR pathogens.

Keywords: Anti-bacterial agent, *Lactobacillus*, Probiotics, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*

INTRODUCTION

The emergence and spread of multidrug-resistant (MDR) Gram-negative bacteria, is of increasing concern due to their intrinsic resistance, rapid acquisition and spread of new resistance mechanisms. Treatment of infections caused by these bacteria is extremely difficult due to the limited therapeutic options available for these pathogens and increases patient mortality and cost of care globally (Koulenti *et al.*, 2019). Therefore, there is an urgent need for alternative treatment options for these MDR strains. Lactobacilli are among the most common probiotics considered to be biological therapeutics, immune-modulating agents and are generally regarded as safe (GRAS). Previous studies have reported probiotic effects on variety of gastrointestinal disorders including lactose tolerance (de Vrese *et al.*, 2001), prevention and alleviation symptoms of traveler's diarrhea (Marteau *et al.*, 2002) and protection against intestinal infections (Reid *et al.*, 2001).

Several mechanisms related to the antimicrobial mechanism of *Lactobacillus* include the following mechanisms: production of inhibitory compounds, immune-stimulation, nutrient competition and competition with pathogenic bacteria for binding sites (Chen *et al.*, 2019). The inhibitory compounds produced by *Lactobacillus* include organic acids (lactic acid, formic acid, acetic acid), ethanol, hydrogen peroxide, fatty acid and antimicrobial peptides (bacteriocins) (Rushdy and Gomaa, 2013; Inglin *et al.*, 2015; Chen *et al*., 2019). By these antimicrobial mechanisms, lactobacilli have shown antimicrobial activity against a wide range of pathogenic bacteria including *Shigella sonnei* and *Shigella flexneri* (Mirnejad *et al.*, 2013), *Salmonalla typhimurium* (Hudault *et al.*, 1997), Carbapenem-resistant *Enterobacteriaceae* (Chen *et al*., 2019), extended-spectrum β-lactamase producing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (El-Mokhtar *et al.*, 2020), uropathogenic *Escherichia coli* (Ghane *et al.*, 2020), *Clostridium* species

*Corresponding author

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(Monteiro *et al.*, 2019), *Staphylococcus aureus* (Kang *et al.*, 2017) and *Helicobacter pylori* (Aiba *et al.*, 1998).

In recent years, a large number of probiotic microorganisms have been introduced and considerable numbers of them are commercially available. However, search for new isolates is still of great interest. Kefir is one of the most important sources of probiotic microorganisms. Previous *in vitro* studies have demonstrated the association of kefir beverage with immunomodulation (Hong *et al.*, 2009), alleviation of lactose intolerance (Hertzler and Clancy, 2003), balance of the intestinal microbiota (Urdaneta *et al.*, 2007) and antimicrobial activity against pathogens (Chifiriuc *et al.*, 2011).

This study aimed to identify suitable lactobacilli strains from kefir that have antibacterial activity against MDR Gram-negative isolates. *In vitro* probiotic properties including acid and bile tolerance, auto-aggregation and co-aggregation with pathogenic bacteria and hemolytic activity were also investigated. This is the first time study to identify antimicrobial property of kefir derived probiotic bacteria against MDR pathogens.

MATERIALS AND METHODS

Isolation of lactobacilli strains

Lactobacilli strains were isolated from kefir grains. Briefly, 20 g of kefir grains were activated by adding 400 mL of sterilized milk and incubating at 21 °C for 24 h. The grains were retrieved by a sterilized strainer and 10 g of activated grains were homogenized in 90 g of sterile saline buffer. Thereafter, serial dilutions were made and each diluted solution was spread plated onto Man Rogosa and Sharpe (MRS) agar (Merk, Germany). The plates were incubated in anaerobic condition (Gaspak EZ, Difco) at 30 °C for 48 h and the resulting colonies were streaked on MRS agar plates to obtain pure colonies. Preliminary identification of the isolates was carried out using Gram staining and catalase test. Gram-positive, catalase negative and rod shape strains were stored in skim milk with 20% glycerol at –80 °C for further experiments.

Clinical isolates

Clinical isolates of *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (10 strains each) were obtained from urine samples of patients who admitted to Milad Hospital and identified by conventional biochemical tests. Antibiotic susceptibility test for clinical isolates was performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). The antibiotic disks used were gentamicin (30 µg), amikacin (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), cefepime (30 µg), levofloxacin (5 µg), ciprofloxacin (30 µg), meropenem (30 µg), imipenem (10 µg) and piperacillin (30 µg) (Mast Diagnostics, UK). In brief, a suspension of each bacterial strains equivalent to 0.5 McFarland standard (approximately 10^8 CFU/mL) was spread onto the surface of Mueller-Hinton agar (MHA) (Merck, Germany) plates. The antibiotic disks were then placed on the agar medium and after incubation at 37 °C for 24 h, the inhibition zone around the disks were measured and interpreted according to CLSI guidelines. Multidrug resistance (MDR) was determined as resistance to at least one agent in three or more antibiotic classes (Magiorakos *et al.*, 2012).

Antibacterial activity of lactobacilli isolates using the well-diffusion method

The antibacterial activity of lactobacilli isolates was assessed primarily by agar well diffusion method. The whole bacteria culture (WBC) and cell-free supernatant (CFS) were tested for their antimicrobial activity. To obtain CFS, a single colony of each lactobacilli strain was inoculated to 20 mL of MRS broth and incubated for 24 h at 30 °C in anaerobic condition. The samples were then centrifuged (Hettich, Germany) at 9000× *g* for 10 min at 4 °C and the supernatant was sterilized using syringe filters (0.22 µm) and used freshly. A 100 µL of overnight culture of clinical isolates equivalent to 0.5 McFarland (10^8 CFU/mL) was spread onto the MHA plate and 6 mm wells were punched with a sterilized Pasteur pipette. Thereafter, 100 µL of WBC and CFS was added to wells and after incubation at 37 °C for 24 h, the diameter of the inhibition zone around the well was measured in millimeters (de Carvalho *et al.*, 2006). MRS broth without CFS was used as negative control. The experiment was performed in triplicates.

Molecular identification of lactobacilli strains

To identify lactobacilli strains, the genomic DNA of each isolate were extracted using SinaPure DNA extraction kit (Sinaclone, Iran) and 16S rRNA gene was amplified using two universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') as described previously (Leite *et al.*, 2015). The PCR products were purified by high pure PCR product purification kit (Sigma Aldrich, USA) and bidirectionally sequenced by dideoxy sanger method in Macrogen Corporation (Seoul, South Korea). The sequences were then aligned using the BLAST search tool (http://www.ncbi.nlm.nih.gov.blast) to find the closest phylogenetic relatives of the sequenced gene. Mega software (version 5) was used to compare consensus sequence and plot dendrogram based on nucleotide sequence similarity. The evolutionary history was deduced by using the Neighbor-Joining method with 1000 replicate bootstrap test and the evolutionary distances were calculated using the maximum composite likelihood method (Tamura *et al.*, 2004).

Broth microdilution test

Broth microdilution test was performed as described previously (Chen *et al*., 2019). Overnight culture of each MDR strains were inoculated (1% v/v) into fresh TSB medium and seeded in 96-well plate. The CFS of each

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selected lactobacilli isolates were prepared and subjected to three different treatments. The first aliquot of CFS was heated at 80 °C for 10 min, the second was neutralized to pH 7 (with 1N NaOH), and the third one received no treatment. A 200 µL of test solution, consisting of 100 µL of the MDR bacterial culture (at final concentration of 10^6 CFU/mL) and 100 µL of one of the CFS aliquots, was added into the wells of microplate. CFS was diluted with MRS broth and applied at different percentages of 10, 20, 30, 40 and 50% in the final volume of 200 µL. Wells containing pathogenic bacteria without CFS was used as negative control. Minimum inhibitory percentage (MIP) was considered as the lowest percentage of CFS that inhibits visible growth of the pathogens. The minimum bactericidal percentage (MBP) was determined by sub-culturing the samples on MHA plates and the highest dilution that yielded no bacterial growth was considered as MBP. The experiment was performed in triplicates.

Kinetic killing assessment

Time-killed kinetic of lactobacilli CFS was carried out using the method proposed by El-Mokhtar *et al.* (2020) with minor modifications. A 100 µL of pathogenic bacteria suspension in Müller-Hinton Broth (MHB) (Merk, Germany) (OD 600 = 0.2) was added to wells of microplate and incubated at 37 °C for 4 h with agitation (180 rpm). Then, CFS at the concentration of 100% was added and the microplates were incubated at 37 °C for 24 h. To assess the viable count of pathogens, aliquots of the samples were taken at different time intervals (0, 4, 8 and 24 h) and inoculated into MHA plates. The plates were then incubated at 37 °C for 24 h and the colony-forming unit (CFU) was measured. Treatments without CFS were used as control. The test was performed in three parallel and independent experiments.

Screening of probiotic properties of lactobacilli isolates

Acid tolerance

Lactobacilli isolates were cultured in MRS broth and after incubation (24 h at 30 °C), the cells were harvested by centrifugation at 7,500× *g* for 5 min at 4 °C. Thereafter, the harvested cells were resuspended in acidic MRS broth (pH 3) and MRS broth at pH 6.5 (control) and incubated at 30 °C. Sampling was performed at time 0 and 3 h and diluted in phosphate buffer saline (PBS) (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl). A 100 µL of diluted samples were spread onto MRS agar plates and incubated at 30 °C for 48 h. Cell viability was evaluated by the plate count method and the results were reported as log CFU/mL (Yadav *et al.*, 2016).

Bile tolerance

Tolerance to bile salts was determined by growing the isolates in broth medium as described previously (Guo *et al.*, 2009). In brief, overnight cultures of lactobacilli isolates were inoculated (1%) into MRS broth containing 0.3% Oxgall (Sigma-Aldrich, USA) and incubated for 9 h at 30 °C. MRS broth without Oxgall was also inoculated in the same way and used as control. The optical density of the cultures at 620 nm was measured every hour. Bile salt tolerance was assessed based on the time required to increase the absorbance at 600 nm by 0.3 units in MRS broth with and without 0.3% Oxgall. The time difference (h) required to increase 0.3 units the absorbance of MRS broth with or without Oxgall was considered as lag time (LT) of the cells to adapt to media containing bile salt.

Auto-aggregation and co-aggregation

Auto-aggregation of LAB strains was determined as described previously (Collado *et al.*, 2008). In brief, LAB strains were cultured in MRS broth and incubated at 37 °C for 24 h. After incubation, the cultures were centrifuged (5000× *g*, 20 min at 4°C) and the cells were resuspended in PBS pH 7 at a concentration of 10^8 CFU/mL. The suspension was then incubated for 24 h at 37 °C and the absorbance at 600 nm was recorded before and after incubation. The auto-aggregation ability of the isolates was calculated with the following formula: $[1 – (A_t/A_0) \times 100]$ where A_t represents the absorbance at 24 h and A_0 absorbance at time 0.

To assess the co-aggregation ability of lactobacilli isolates with MDR isolates, bacterial cells from an overnight culture were harvested by centrifugation at 5000× *g* for 20 min at 4 °C and suspended in PBS pH 7 (10^8 CFU/mL). Bacterial suspension of MDR strains were similarly prepared and mixed with equal volumes (500 mL) of the cultures of the lactobacilli strains. The mixture was then incubated at 37 °C for 24 h and the absorbance (OD600 nm) was measured after 24 h at 37 °C. The co-aggregation percentage was calculated with the following formula: $[(A_{pathog} + A_{LAB})/2 – (A_{mix})/(A_{pathog} + A_{LAB})/2] \times 100$, where A_{pathog} and A_{LAB} represent the OD of tubes containing pathogens or LAB strains respectively, and A_{mix} indicates the OD of the mixture after 24 h (García-Cayuela *et al.*, 2014).

Hemolytic Activity

All the selected isolates were streaked onto blood agar plates containing 5% (w/v) sheep blood and incubated at 37 °C for 48 h. After incubation, the plates were examined for α-hemolysis, β-hemolysis or non-hemolytic activities. Greenish or clear zone around the isolates were recorded as α-hemolysis or β-hemolysis respectively, and no hemolysis was scored as negative (-).

Sugar fermentation

Acid production from carbohydrates (fructose, lactose, glucose, sucrose, mannitol, trehalose, mannose, maltose) was assessed as described previously (Ricciardi *et al.*, 2005). A 100 g/L solution of each sugar was prepared and sterilized using syringe filters (0.22 µm). A 1 mL of

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mentioned solution was added to 9 mL of basal MRS broth medium (without glucose and meat extract and with 0.16 g/L bromocresol purple, pH 7.0) and was distributed (180 µL) into the wells of a microtitre plate. Overnight culture of lactobacilli isolates was centrifuged (10000× *g*, 5 min), resuspended in sterile saline, and added (20 µL) to the well of microtitre plates. The plates were then sealed and incubated for 48 h at 30 °C in anaerobic condition. Any change in color from purple to red was considered as positive reaction.

Statistical Analysis

All tests were performed as three parallel independent experiments and the results were expressed as mean ± standard deviations (SD). SPSS 20 following Tukey's test was carried out to compare means. $P<0.05$ was set as significance.

RESULTS

Antimicrobial susceptibility of pathogenic clinical isolates

The antimicrobial susceptibility testing of *E. coli*, *P. aeruginosa* and *K. pneumoniae* (10 strains each) showed that the isolates were resistant to most antibiotics including amikacin, gentamicin, cefepime, ceftazidime and ceftriaxone (Table 1). All the isolates were MDR and the most effective antibiotic was found to be imipenem.

Isolation of lactobacilli strains and their antimicrobial activity

In our study, 21 Gram-positive, catalase-negative and rod shape strains were isolated from kefir grains and assessed for antimicrobial activity against MDR strains by using agar well diffusion method. The mean values of inhibition zones of lactobacilli isolates against MDR strains of *E. coli*, *P. aeruginosa* and *K. pneumonia* (10 isolates each) is presented in Figure 1. Most lactobacilli strains exhibited high antibacterial activity against clinical isolates. However, the level of antibacterial activity was different depending on the lactobacilli strains.

In the present study, WBC represented a higher antibacterial activity than CFS against all clinical isolates. Based on the antimicrobial activity results, four strains (A31, B35, S20 and S25) that their WBC and CFS displayed the greatest antimicrobial activity against all pathogens were selected for further studies. An approximately 1500 bp region of 16S rRNA gene of each selected isolates was amplified using primers 27F and 1492R and subjected to sequencing. Then, the isolates were identified based on 16S rDNA sequences and their sequences were deposited in GenBank. Molecular identification revealed that the isolates were related to *Lactobacillus rhamnosus* and *Lactobacillus paracasei* (Table 2). The phylogenetic relationship of selected lactobacilli isolates among the members of genus *Lactobacillus* is shown in Figure 2.

Table 1: Antimicrobial resistance profile of clinical isolates used in this study.

Microorganism	Antimicrobial resistance profile	Isolates code
E. coli (n=10)	AK-MER-CRO-CPM-LEV-CIP	E21, E28
	AK-CAZ-CPM-PRL-LEV-CIP	E32, E33
	AK-GM-CAZ-PRL-LEV-CIP	E35, E40
	GM-CAZ-CPM-PRL-LEV-CIP	E42
	AK-GM-CAZ-CRO-CPM-PRL-LEV-CIP	E65
	AK-GM-MER-CAZ-CRO-CPM	E67
	AK-GM-MER-CAZ-CRO-CPM-PRL-LEV-CIP	E81
P. aeruginosa (n=10)	AK-GM-MER-CPM-CAZ-LEV-PRL-CRO	P18, P21, P22
	AK-GM-CPM-CAZ-CIP-PRL-CRO	P24, E28, P29
	AK-CIP-LEV-CRO-FOX-PRL-IMI	P30, E33
	AK-CPM-CAZ-CIP-LEV-PRL-CIP	P36
	AK-GM-MER-CPM-CAZ-CIP-PRL	P43
K. pneumoniae (n=10)	AK-GM-MER-CPM-CAZ-LEV-PRL-CRO-FOX	K10, K13
	GM-AK-CPM-CAZ-CIP-PRL-CRO-FOX	K21, K45, K49
	GM-AK-CIP-LEV-CRO-FOX-IMI	K61
	GM-AK-CPM-CAZ-CIP-LEV-PRL-FOX	K80, K89
	GM-AK-MER-CPM-CAZ-CIP-LEV-PRL	K91, K93

Abbreviations: CAZ: Ceftazidime; CRO: Ceftriaxone; IMI: Imipenem; MER: Meropenem; AK: Amikacin; GM: Gentamicin; CPM: Cefepime; LEV: Levofloxacin; CIP: Ciprofloxacin; PRL: Piperacillin; FOX: Cefoxitin.

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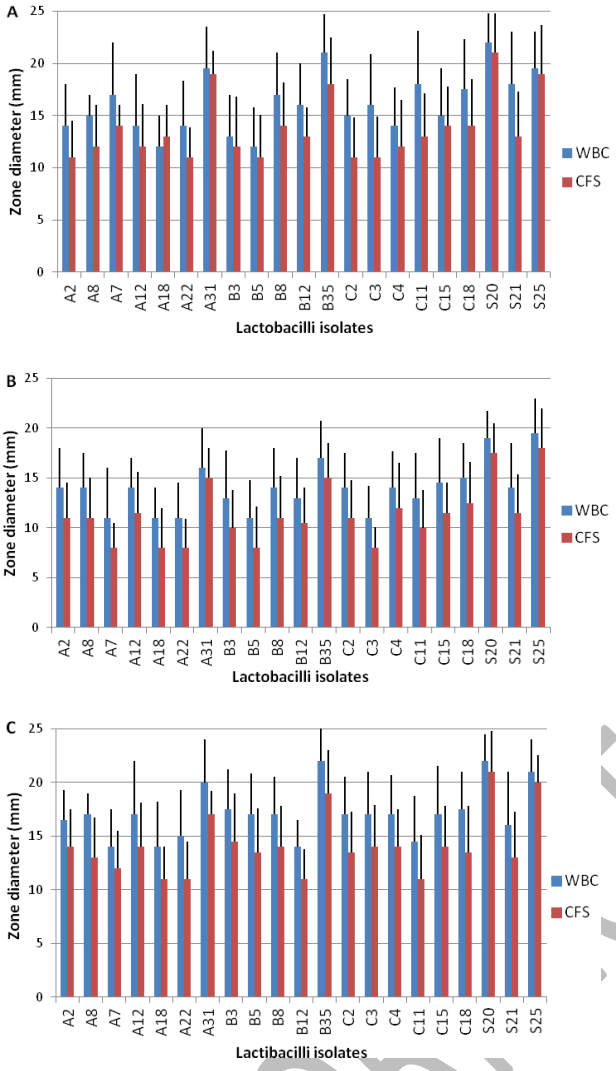

Figure 1: The mean values of inhibition zones of *Lactobacillus* isolates against MDR strains by well diffusion method. Each mean value has yielded from the zone of inhibition diameters of 10 of (A) *K. pneumoniae*, (B) *P. aeruginosa* and (C) *E. coli* clinical isolates respectively. WBC: whole bacteria culture; CFS: cell-free supernatant.

Table 2: The GenBank accession numbers of Kefir lactobacilli isolates characterized by 16S rRNA gene sequencing.

Isolates	The closest relative	Accession no.	Identity (%)
S20	*L. paracasei*	MH819524	100
S25	*L. paracasei*	MH819548	99.75
A31	*L. paracasei*	MH819523	100
B35	*L. rhamnosus*	MH333150	99.85

Minimum inhibitory percentage (MIP) and minimum bactericidal percentage (MBP) assessment

The results of MIP and MBP test of untreated CFS of four *Lactobacillus* strain against MDR isolates are shown in Table 3. The MIP and MBP of these selected lactobacilli isolates were in the range of 10-30% and 20-50%, respectively. In order to investigate on the nature of the inhibitory substances secreted by each lactobacilli strain, CFSs were subjected to different treatments. The antimicrobial activity of CFSs did not alter after heating up to 80 °C for 10 min (data not shown). The CFSs of all 4 lactobacilli isolates after 24 h grown in MRS broth had a final pH in the range of 3.72-3.74 (Table 3). In order to determine whether the antimicrobial activity of CFSs is due to the low pH, the pH of CFSs was further set to 7 and its antibacterial activity assessed. The antibacterial activity of all CFSs against all MDR isolates was completely disappeared after neutralizing pH to 7 (data not shown).

Kinetic killing assessment

The kinetic killing curve of CFS of selected lactobacilli isolates in suppressing the growth of MDR *E. coli* (E81), *K. pneumoniae* (K10) and *P. aeruginosa* (P18) are presented in Figure 3. These MDR strains were randomly selected from isolates that were resistant to a larger number of antibiotics. The growth of MDR isolates was decreased after incubation with CFS of all lactobacilli strains in all tested time intervals compare to cultures without CFS. The CFS of all lactobacilli isolates significantly inhibited growth of *E. coli* (E81) and *K. pneumoniae* (K10) MDR isolates after 24 h (all $P<0.001$) and induced about 2.9-3.5 log reduction in the growth of E81 and 3.5-4.5 log reduction of K10 compared to cultures without CFS (Figure 3A and 3C). *P. aeruginosa* (P18) was also inhibited significantly over 24 h incubation under CFS treatment of S20, S25 (both $P<0.001$), A31 and B35 (both $P<0.05$). CFS of strains S20 and S25 exhibited better inhibitory activity against MDR *P. aeruginosa* (P18) than other lactobacilli strains and resulted in 2.5 and 2.8 log reduction in viability, respectively (Figure 3B).

Acid and bile tolerance of selected lactobacilli strains

Lactobacilli strains were examined for acid tolerance at pH 3 by using viable cell count method. All the isolates showed tolerance to pH 3 and the viable counts of all isolates were greater than 10^6 CFU/mL after 3 h exposure of acid (Table 4).

Bile salt tolerance results showed that all the isolates could grow at 0.3% of bile salts, although the lag time was different between the isolates. Among the isolates, S20 and B35 exhibited the shortest lag time and so were more resistant to bile salts ($P<0.05$) (Table 4).

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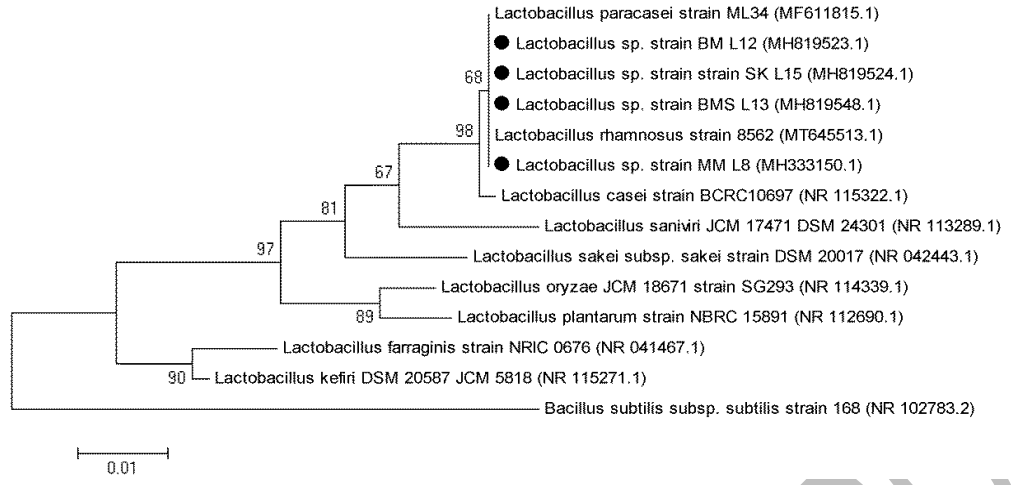

Figure 2: Phylogenetic tree based on the 16S rDNA sequences of selected lactobacilli strains. Accession numbers are in parentheses.

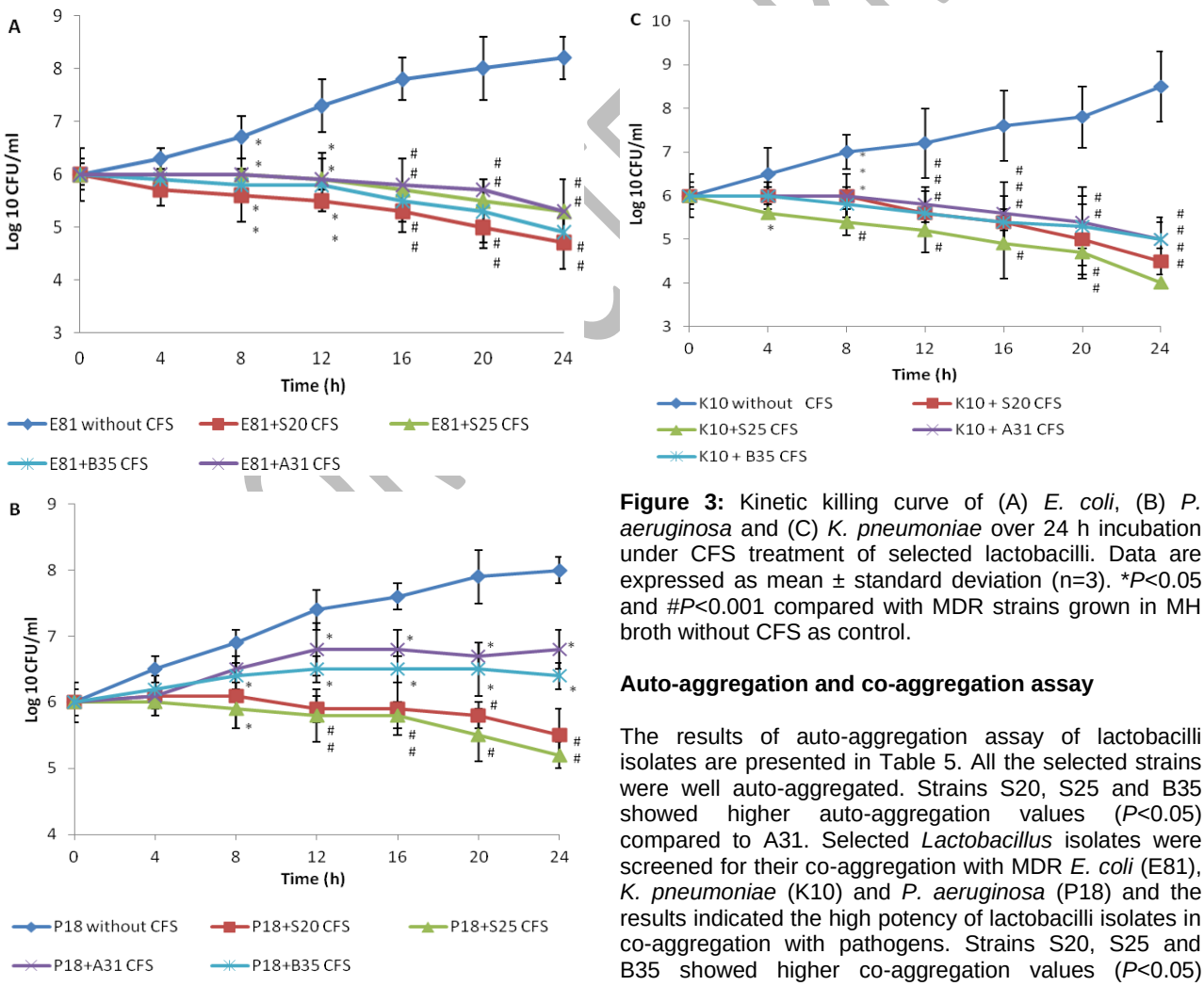

Figure 3: Kinetic killing curve of (A) *E. coli*, (B) *P. aeruginosa* and (C) *K. pneumoniae* over 24 h incubation under CFS treatment of selected lactobacilli. Data are expressed as mean ± standard deviation (n=3). **P*<0.05 and #*P*<0.001 compared with MDR strains grown in MH broth without CFS as control.

Auto-aggregation and co-aggregation assay

The results of auto-aggregation assay of lactobacilli isolates are presented in Table 5. All the selected strains were well auto-aggregated. Strains S20, S25 and B35 showed higher auto-aggregation values (*P*<0.05) compared to A31. Selected *Lactobacillus* isolates were screened for their co-aggregation with MDR *E. coli* (E81), *K. pneumoniae* (K10) and *P. aeruginosa* (P18) and the results indicated the high potency of lactobacilli isolates in co-aggregation with pathogens. Strains S20, S25 and B35 showed higher co-aggregation values (*P*<0.05) compared to A31 (Table 5).

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Table 3: Minimum inhibitory percentage (MIP) and minimum bactericidal percentage (MBP) of untreated CFS of four *Lactobacillus* strain against MDR *E. coli*, *P. aeruginosa* and *K. pneumoniae*.

Isolates	pH	E21	E28	E32	E33	E35	E40	E42	E65	E67	E81
S20	3.72	10/20	10/30	10/20	10/30	10/30	10/30	10/20	10/20	10/20	10/20
S25	3.82	10/30	10/40	10/40	10/40	10/40	10/40	10/30	10/30	10/30	10/30
A31	3.99	10/30	10/20	10/20	10/20	10/20	10/30	10/30	10/20	10/30	10/30
B35	3.84	10/40	10/40	10/30	10/30	10/30	10/40	10/40	10/40	10/40	10/40
	pH	P18	P21	P22	P24	P28	P29	P30	P33	P36	P43
S20	3.72	20/30	20/30	20/40	20/40	20/30	20/30	20/40	20/40	20/40	20/50
S25	3.82	20/30	20/40	20/40	20/40	20/30	20/40	20/40	20/30	20/30	20/40
A31	3.99	30/50	30/40	30/40	30/50	30/50	30/40	30/50	30/40	30/50	30/50
B35	3.84	30/50	30/50	30/40	30/40	30/50	30/50	30/40	30/50	30/40	30/40
	pH	K10	K13	K21	K45	K49	K61	K80	K89	K91	K93
S20	3.72	10/20	10/20	10/30	20/30	20/30	10/20	10/30	10/20	10/30	20/30
S25	3.82	10/30	10/30	10/20	20/30	10/30	10/20	10/20	10/30	20/30	20/30
A31	3.99	10/20	10/20	10/20	20/30	20/30	10/20	20/30	10/20	20/30	20/30
B35	3.84	10/20	10/20	10/30	10/30	10/30	20/20	10/30	10/30	10/30	20/30

Values are expressed as MIP/MBP (%).

Table 4: Acid and bile tolerance of selected lactobacilli isolates.

Isolates	Cell viability (log_{10} CFU/mL)				Bile tolerance (Time needed for increasing 0.3 units)		
	pH 6.5		pH 3		MRS (h)	MRS + 0.3% bile (h)	Lag time (h)
	0 h	3 h	0 h	3 h			
S20	7.85 ± 0.85^{d}	8.20 ± 0.10^{a}	7.80 ± 0.42^{b}	7.40 ± 0.31^{a}	3.73 ± 0.01^{b}	4.73 ± 0.01^{c}	1.00 ± 0.02^{c}
S25	7.95 ± 0.42^{b}	8.36 ± 0.15^{a}	7.89 ± 0.12^{b}	6.49 ± 0.19^{c}	4.44 ± 0.02^{a}	5.95 ± 0.05^{b}	1.51 ± 0.05^{b}
A31	7.89 ± 0.25^{c}	8.23 ± 0.05^{a}	7.85 ± 0.35^{b}	6.97 ± 0.23^{b}	4.43 ± 0.15^{a}	6.43 ± 0.11^{a}	2.00 ± 0.17^{a}
B35	8.22 ± 0.39^{a}	8.50 ± 0.17^{a}	8.10 ± 0.28^{a}	6.30 ± 0.08^{d}	3.70 ± 0.05^{b}	4.70 ± 0.06^{c}	1.00 ± 0.04^{c}

Data are represented as mean ± SD of three independent experiments. Different subscript letters in each column represents significant difference.

Table 5: Auto-aggregation and co-aggregation values of lactobacilli strains with MDR isolates.

Isolates	Auto-aggregation (%)	Co-aggregation with MDR isolates (%)		
		E 81	P 18	K 10
S20	63.3 ± 2.0^{a}	62.0 ± 7.2^{ab}	53.7 ± 10.0^{ab}	65.3 ± 8.3^{a}
S25	55.3 ± 3.0^{ab}	73.3 ± 7.6^{a}	69.3 ± 8.1^{a}	49.7 ± 5.7^{ab}
A31	37.3 ± 2.0^{c}	39.3 ± 8.1^{c}	45.0 ± 11.0^{c}	39.0 ± 4.5^{b}
B35	46.7 ± 3.5^{b}	51.7 ± 7.5^{bc}	62.0 ± 6.6^{ab}	53.3 ± 6.6^{ab}

Data are represented as mean ± standard deviation of three parallel independent experiments. Different subscript letters in each column represents significant difference.

Table 6: Sugar fermentation profile of the selected lactobacilli isolates.

Isolates	Carbohydrates							
	fructose	lactose	glucose	sucrose	mannitol	trehalose	mannose	maltose
S20	+	+	+	+	+	+	+	+
S25	+	+	+	+	+	-	+	+
A31	+	-	+	+	-	+	+	+
B35	-	+	+	+	+	+	+	+

+: positive reaction; -: negative reaction.

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Carbohydrate fermentation and safety assay

The sugar fermentation profile of the isolates is shown in Table 6. Among the isolates, S20 could ferment all tested sugar. Besides, none of the selected lactobacilli isolates showed hemolytic activity.

DISCUSSION

Today, the demand for new lactobacilli strains with antimicrobial properties against MDR pathogens is increasing, and kefir could be the best source for obtaining these bacteria due to its complex microbiota. In the preset study, lactobacilli strains were isolated from kefir grains and their antimicrobial activity and probiotic properties were assessed.

Previous studies reported the antimicrobial activity of *Lactobacillus* CFS against a wide range of microorganisms including *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* (Muhammad *et al.*, 2019), *Shigella sonnei*, *Shigella flexneri* (Mirnejad *et al*., 2013) and *Vibrio* spp. (Kaur *et al.*, 2018). In the present study, all the MDR strains were inhibited by lactobacilli strains. However, the level of inhibitory effect was different depending on the lactobacilli strains. In our study, WBC represented a higher antibacterial activity than CFS against all clinical isolates. These results are in accordance with the results obtained by Ołdak *et al.* (2017), where they found significant differences in the antagonistic activity of the CFS and WBC of *L. plantarum* against *L. monocytogenes* strains. Higher levels of antagonistic activity of WBC may be due to the presence of live lactobacilli strains capable of producing active antimicrobial compounds.

In the present research, four lactobacilli strains including three *L. paracasei* strains (S20, S25 and A31) and one *L. rhamnosus* strains (B35) showed good inhibitory activity against all MDR strains. The antimicrobial activity of CFS of these isolates was further assessed by broth microdilution assay and time-kill test against MDR isolates. Previous studies also proved the antibacterial potential of *L. paracasei* and *L. rhamnosus* against carbapenem-resistant *Enterobacteriaceae* (Chen *et al*., 2019). The antimicrobial activity of CFS may be attributed to the production of bacteriocins, organic acids and hydrogen peroxide (Muhammad *et al*., 2019). In this study, when the pH of CFS was neutralized in MIP/MBP experiments the antimicrobial activity of selected lactobacilli strains was completely abolished. Interestingly, the antimicrobial activity of CFSs was not inhibited by heating the CFSs up to 80 °C. These findings indicate that the inhibitory activity of CFS is due to the acid production by lactobacilli isolates and not bacteriocin-like substances. It has been reported that the acidity of the lactic acid produced by lactobacilli can disrupt the outer membrane of Gram-negative bacteria (Alakomi *et al.*, 2000). In time-killed study, the CFS of all the lactobacilli isolates showed reduction in growth of MDR strains. In a study conducted by El-Mokhtar *et al*. (2020), *Lactobacillus* isolated from yogurt induced at least 1.5 log reduction in growth of *K. pneumoniae* and *P. aeruginosa*. Compared to their results, the lactobacilli isolates obtained in this study induced higher reduction in growth of MDR isolates of *K. pneumoniae* and *P. aeruginosa* (3.5-4.5 and 2.5-2.8 log reduction, respectively). These high rates of growth reduction may be due to the low pH of CFS (Table 3) which can inhibit the growth of MDR strains.

The isolates were further assessed for their probiotic potential. In our study, all the isolates showed tolerance to pH 3 and the viable counts of all isolates were greater than 10^6 CFU/mL after 3 h exposure. Tolerance to acidic pH by lactobacilli isolates from kefir grains was also reported in previous studies (Zheng *et al.*, 2013; Leite *et al*., 2015). Tolerance to acidic pH is a major selection criterion because probiotic strains should be able to survive in the upper gastrointestinal tract during transit to the intestinal tract (Bao *et al.*, 2010). Bile salt tolerance is a required property for colonization of bacteria in small intestine (Yadav *et al*., 2016). In our study, all the isolates could grow at 0.3% of salts, a similar concentration to that present in the intestinal tract.

In this study, all the strains were well auto-aggregated. Auto-aggregation is an exopolysaccharides mediated process by which probiotic bacteria interact physically with each other and adhere to surfaces (Sorroche *et al.*, 2012). The auto-aggregation ability of probiotic strains helps them to maintain in the intestine (Rickard *et al.*, 2003).

Selected *Lactobacillus* isolates were also screened for their co-aggregation with pathogens and the results showed that all the selected *Lactobacillus* isolates were able to co-aggregate with MDR isolates. Co-aggregation of *Lactobacillus* strains with pathogenic bacteria plays an important role in preventing colonization of pathogens on biotic or abiotic surfaces, and thus interferes with the ability of pathogens to infect host and inhibit the colonization of food borne pathogenic bacteria (García-Cayuela *et al.*, 2014). In the present study, the selected lactobacilli strains exhibited high co-aggregation results and strain S25 showed the highest co-aggregation value (73.3% with *E. coli* strain E81 and 69.3% with *P. aeruginosa* strain P18). Interestingly, this strain also showed high antimicrobial activity on pathogenic strains. In this case, co-aggregation can increase the antimicrobial efficiency of *Lactobacillus*, as it makes *Lactobacillus* in close contact with pathogenic bacteria. Co-aggregation can also increase the concentration of secreted inhibitory substances (Kaewnopparat *et al.*, 2013). Hence, the lactobacilli strains that well co-aggregate with pathogenic bacteria are of special interest with regard to their potential for safe therapy of infectious disease.

In our study, lactobacilli isolates were able to ferment the tested sugars. However, the sugar fermentation profile differed between isolates. Similar to our results, Abedi *et al*. (2013) reported differences in metabolic activity between tested lactobacilli isolates. In the present study, all selected lactobacilli isolates presented no

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hemolytic activity, and this is in accordance with previous safety requirements reported by Angmo *et al*. (2016). Our findings are in agreement with the results of Oh and Jung (2015) who determined the hemolytic activity of *Lactobacillus* species isolated from traditionally fermented millet-based alcoholic beverages and revealed no hemolytic activity of probiotics. Lack of hemolytic activity is an important criterion in the selection of probiotic strains, because such strains are non-virulence (Yasmin *et al*., 2020).

CONCLUSION

Our results indicated that all selected lactobacilli strains had broad spectrum activity against MDR strains. The isolates have also been well auto-aggregated and co-aggregated with pathogenic bacteria. To our best knowledge, this is the first study describing the antibacterial activity of kefir derived *Lactobacillus* strains against MDR isolates. Our results offer a promising role of lactobacilli in prevention and treatment of MDR isolates. However, *in vivo* experiments are needed to clarify this issue.

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