


Antagonistic Activities of Cell-Free Supernatants of Lactobacilli Against Extended-Spectrum β -Lactamase Producing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

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Mohamed A El-Mokhtar ¹
Khaled M Hassanein¹
Ahmed S Ahmed¹
Gamal FM Gad²
Mohamed M Amin³
Osama FE Hassanein⁴

¹Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt;

²Department of Microbiology and Immunology, Faculty of Pharmacy, Minia University, Minia, Egypt; ³Department of Microbiology and Immunology, Faculty of Medicine, Aswan University, Aswan, Egypt; ⁴Drug Research Center, Assiut University, Assiut, Egypt

Aim: This study aimed to describe the inhibitory activity of cell-free supernatants (CFS) of lactobacilli against extended-spectrum β -lactamase (ESBL)-producing *Klebsiella pneumoniae* (*K pneumoniae*) and *Pseudomonas aeruginosa* (*P aeruginosa*).

Material and Methods: Pathogenic clinical strains of *K pneumoniae* and *P aeruginosa* were isolated from urine samples and selected for investigation. Anti-bacterial activities of the CFS of lactobacilli were assessed by agar well diffusion, MTT assay, as well as time-kill tests. In addition, the antibiofilm characteristics were analyzed by the microplate method against fresh and 24 h-old biofilms. The ability of CFS to interfere with bacterial invasion was analyzed by flow cytometry.

Results: Although all tested strains were ESBL producers and showed a multidrug-resistant phenotype, the CFS displayed a high anti-ESBL activity with inhibition zone diameters greater than 13 mm in the agar well diffusion assays against both pathogens. The growth kinetics of *K pneumoniae* and *P aeruginosa* were dramatically decreased in the presence of the CFS. The CFS not only inhibited the biofilm formation by these pathogens but also was able to remove the 24-h formed biofilms. The invasion abilities of FITC-labelled *K pneumoniae* decreased from 30.3% \pm 7 to 15.4% \pm 5 and invasion of FITC-labelled *P aeruginosa* was reduced from 36.9% \pm 7 to 25.2% \pm 5.

Conclusion: CFS of lactobacilli exhibit anti-ESBL activities, which suggests its potential application for controlling or preventing colonization of infections caused by ESBL-producing bacteria.

Keywords: lactobacilli, antibiofilm, ESBL, *K pneumoniae*, *P aeruginosa*

Introduction

Klebsiella pneumoniae (*K pneumoniae*) and *Pseudomonas aeruginosa* (*P aeruginosa*) are Gram-negative opportunistic pathogens that can cause severe nosocomial infections such as bacteremia, pneumonia, urinary tract infections and soft tissue infections, particularly in immune-compromised individuals.¹ These pathogens are well-known for their ability to develop and transfer antibiotic resistance determinants such as the production of extended-spectrum β -lactamase (ESBL), which confers resistance to β -lactam antibiotics, particularly to third-generation cephalosporins.² The spread of ESBL producing Gram-negative bacilli has increase critically worldwide and is one of the most growing problems of antibiotic resistance and leaves only limited treatment options for clinicians.³

Correspondence: Mohamed A El-Mokhtar
Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut 71515, Egypt
Tel +20 122 111 53 13
Email elmokhtar@aun.edu.eg

Moreover, treatment of serious infections with these bacteria is extremely difficult due to co-resistance to multiple antibiotics.⁴ Their pathogenicity is multifactorial, including LPS, capsule, adherence factors and exotoxins, and till now, no effective vaccines are developed for protection from these pathogens.⁵ A common virulence strategy for both pathogens is the ability to form biofilms. Bacteria in biofilms are not only resistant to immune defense mechanisms but also to many antibiotics due to the production of a protecting extracellular polymer matrix.^{6,7} Therefore, there is an urgent need for new treatment strategies for these critical groups of pathogenic bacteria.

Lactobacilli is one of the most common probiotics that is generally recognized as safe (GRAS) biological therapeutic agent and is also used to boost the host immune responses. There are different mechanisms by which lactobacilli can exert their antimicrobial activity, including the production of inhibitory compounds, immune stimulation, competition with pathogenic bacteria for the receptor binding, and competition on nutrients. The inhibitory compounds produced by lactobacilli include organic acids such as lactic acid, acetic acid, and formic acid or bacteriocins.^{8,9} Through these antimicrobial mechanisms, lactobacilli have demonstrated antagonistic activities against different pathogenic bacteria, including carbapenem-resistant Enterobacteriaceae,¹⁰ *Escherichia coli*,¹¹ *Helicobacter pylori*,¹² *Salmonella*,¹³ *Shigella*,¹⁴ *P aeruginosa*,¹⁵ and *Staphylococcus aureus*.¹⁶

However, no previous studies have assessed the antimicrobial activity of lactobacilli against ESBL-producing *K pneumoniae* or *P aeruginosa*. Thus, in this study, we aimed to describe the potential antagonistic activities of lactobacilli's CFS on ESBL-producing *K pneumoniae* and *P aeruginosa*.

Materials and Methods

Collection of Samples and Enrichment

Lactobacilli were isolated from plain yogurt samples that were prepared from cow milk and purchased from a local dairy shop in Assiut, Egypt. One gram of the yogurt sample was inoculated in de Man Rogosa Sharpe (MRS) broth followed by streaking on the MRS agar (Thermo Fisher Oxoid, UK). Plates were incubated anaerobically at 37°C for 48 hrs and the resulting colonies were purified by repeated subculture and preliminarily identified as *Lactobacillus spp.* based on Gram staining, morphology, sporulation and biochemical test results.^{17,18}

Identification of *Lactobacillus Spp.* And Preparation of Cell-Free Supernatants

To identify the species of the isolated bacteria, we sequenced the 16S ribosomal RNA gene. Bacterial genomic DNA was purified from 5 mL overnight MRS broth cultures according to the manufacturer's instructions (PureLink[®] Genomic DNA, Life Technologies Corp., USA). The 16S ribosomal RNA gene was amplified using Lac16f (5'-AGAGTTTGATC CTGGCTCAG-3') and Lac16r (5' -CTACGGCTACCTTG TTACGA-3') primers.¹⁹ The PCR conditions were as follows: initial denaturation at 95°C for 2 mins, followed by 40 cycles of 2 mins at 95°C, 20 S at 49°C and 1 min at 72°C, and a final extension at 72°C for 5 min. The purified products were sequenced in Macrogen Corporation (Seoul, South Korea). Sequences were identified using the NCBI GenBank database using the BLAST search tool (<http://www.ncbi.nlm.nih.gov/blast>) to find the closest match.

For the preparation of cell-free supernatants of lactobacilli, 15 mL of MRS broth was inoculated with single separated colonies of lactobacilli and incubated at 37°C for 24 h. After incubation, samples were centrifuged at 6000 xg for 15 mins, and supernatants were filter-sterilized through a 0.22 µm filter (Millipore Inc., Billerica, USA) and used freshly.

Bacterial Strains and Culture Conditions

To exclude the possibility that the effect of CFS was strain-dependent, 15 different strains of *K pneumoniae* and another 15 different strains of *P aeruginosa* were tested. *K pneumoniae* and *P aeruginosa* were isolated from urine samples of patients suffering from UTI admitted to the Urology Unit, Assiut University hospitals. The identity of these isolates was determined using the API 20E and API20NE identification system (bioMérieux, France). Testing the production of ESBL is described in the next section. For preparation of bacterial suspensions, separate fresh colonies were inoculated into Muller Hinton Broth (MHB; Thermo Fisher Oxoid, UK) and cultured overnight at 37°C. Cell density was determined by measuring the optical density at 600 nm (OD600) using a spectrophotometer (Epoch, USA). These clinical isolates were used to test the antimicrobial and antibiofilm activities of the probiotic supernatants.

Antibiotic Susceptibility Testing

Susceptibility of *K pneumoniae* and *P aeruginosa* isolates to different antibiotics including, Ampicillin, Amoxicillin, Aztreonam, Cefepime, Cefotaxime, Cefoperazone,

Ceftazidime, Ceftriaxone, Imipenem, Meropenem, Gentamicin, Amikacin, Amoxicillin/clavulanic acid, Trimethoprim/sulphamethoxazole were investigated by using Kirby-Bauer disk diffusion method and diameters of inhibition zones were measured and compared with the zones reported by CLSI.²⁰ In addition, bacteria were tested for ESBL production by initially screening the isolates for reduced susceptibility to ceftazidime, ceftriaxone, and cefotaxime. Then ESBL production was confirmed using the combined disc synergy testing between ceftazidime versus ceftazidime-clavulanate and cefotaxime versus cefotaxime-clavulanate where ESBL production was indicated by a ≥ 5 mm increase in the inhibition zone diameter for the antimicrobial agent tested in combination with β -lactamase inhibitor versus its zone when tested alone.²¹

Assessment of the Antibacterial Activity Using the Well-Diffusion Method

The antimicrobial activity of supernatants isolated from lactobacilli was evaluated initially according to the agar well diffusion assay. Mueller Hinton agar plates (Oxoid, USA) were swabbed on the surface with cultures of 15 different pathogenic ESBL-producing *K pneumoniae* or *P aeruginosa* strains adjusted to approximately 10^5 CFU/mL. Then, 5 mm diameter wells were prepared and CFS (100 μ L) was added in the wells. After incubation at 37°C for 24 h, the diameter of the inhibition zone around the well was measured.²² A negative control that consisted of MRS broth without added CFS was included.

Effect of CFS on the Viability of the Pathogenic Bacteria

The impact of CFS on the viability of ESBL-producing *K pneumoniae* and *P aeruginosa* was evaluated using the MTT assay (Promega, USA). Briefly, *K pneumoniae* and *P aeruginosa* (15 strains each) were sub-cultured in LB medium at 37°C overnight. After reaching confluence ($OD_{600}=0.5$), 100 μ L of each culture was added per well in a 96-well plate, followed by the addition of 100 μ L lactobacilli CFS or MRS medium alone (negative control). The cultures were incubated at 37°C for 6 h then 5 μ L MTT solution (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega, USA) was added and incubated for 1 h at 37°C in the dark. Supernatants were carefully discarded, and wells were washed with 250 μ L PBS then the formed crystals were dissolved using 100 μ L of a solubilizing agent. The developed color was measured at 570 nm using

a microplate reader (Epoch, USA). The absorbance values were expressed as the mean percentage of viability relative to control untreated samples.

Kinetic Growth of Pathogenic Bacteria in Lactobacilli CFS-Containing Media

Suspensions of *K pneumoniae* or *P aeruginosa* (15 strains each) in MHB of bacteria (100 μ L) with $OD_{600}=0.2$ were added to wells of 96-well plate and incubated at 37°C for 1–3 h with shaking at 200 r/min. CSF was added at a concentration of 100% and the number of viable bacteria was evaluated by plating out the broth-containing bacteria on the surface of MH agar at 0, 4, 8, 12, 16 and 24 h after incubation and counting the number of CFUs.

Biofilm Removal Activity of Lactobacillus CFS

Testing the biofilm formation was performed using a 96-well microtiter plate as previously described with some modification.²³ Briefly, overnight cultures of ESBL-producing *K pneumoniae* and *P aeruginosa* (15 strains each) were diluted 1:100 into 15 mL of MHB supplemented with 2% w/v sucrose (Sigma-Aldrich, USA), in presence or absence of different concentrations of probiotic supernatants. Cultures were incubated at 37°C for 24 h. After incubation, the developed biofilm was washed 3 times with 200 μ L of distilled water and allowed to air dry. Then, 100 μ L of 0.2% crystal violet (Merck KGaA, Germany) were added to each well, and the plate was incubated for 20 min to allow for biofilm staining. Wells were washed three times with distilled water, air-dried, and treated with 200 μ L of 95% ethanol (Sigma-Aldrich, USA) to dissolve the crystal violet crystals. The plate was incubated for 30 mins and the intensity of the crystal violet was measured at 570 nm using a microplate reader (Epoch, USA). The ability of CFS to affect biofilm formation was measured by comparing the absorbance values of the CFS treated wells versus untreated control wells. To test the ability of CSF to remove the formed biofilms, the same procedures described above were employed, but before the staining step, different aliquots of CFS were gently added to the overnight incubated cultures and then further incubated for 30 min at room temperature then the strength of the biofilm was re-evaluated. Each sample was analyzed in triplicate. To estimate the reduction percent of CFS, the below formula was applied:

$$\begin{aligned} & \text{Percentage of reduction in biofilm} \\ & = [(C - B) - (T - B)] / (C - B) \times 100 \end{aligned}$$

Where C is OD_{570nm} of wells containing bacterial cells without CFS treatment, B is OD_{570nm} of negative controls and T is OD_{570nm} of wells treated with CFS.

Invasion Assay by Flow Cytometry

ESBL-producing *K pneumoniae* and *P aeruginosa* were first FITC labeled. To do that, overnight *K pneumoniae* and *P aeruginosa* cultures in MHB were prepared. Cells were pelleted, washed in sterile saline and then resuspended in 0.1M NaHCO₃ buffer (pH 9), supplemented with 100 µg/mL FITC (Sigma Aldrich, Germany) and incubated for 30 min with constant agitation in the dark. Bacteria were then washed with saline, fixed using 0.5% formaldehyde in PBS for 30 mins, washed and resuspended in PBS supplemented with 1% bovine serum albumin (BSA). 293 cells (kindly provided by tissue culture facility of VACERA, Cairo) were seeded to 24-well plates at a density of 5x10⁴ cells/well. Before the experiment, cells were washed with PBS and maintained in 1 mL of DMEM medium (Gibco), supplemented with 10 mM HEPES and 5% BSA. Cells were treated with FITC-labelled bacteria at an MOI of 10 and incubated at 37°C for 6 h. To test the effect of CFS of lactobacilli, 1 mL of CFS was added to test wells and the broth was added to control cells. Gentamycin (20 mg/mL) was added 20 min before the end of the incubation period to lyse any remaining extracellular bacteria. At the end of the incubation, cells were washed with PBS, trypsinized, and analyzed on a FACS CALIBUR (Becton Dickinson) flow cytometer to determine the percentage of the intracellular bacteria. The effect of CFS on the invasion of *K pneumoniae* and *P aeruginosa* to 293 cells was assessed by comparing the invasive abilities of the microbes in the presence or absence of CFS.

Statistical Analysis

The Student's *t*-test was used to evaluate the effect of CFS treatment. The significance level was set at P<0.05 for all comparisons. All statistical analyses were performed using SPSS version 16.

Results

Isolation and Identification of Lactobacilli

Different yogurt samples were collected from the milk vendors. For presumptive phenotypic identification, samples were cultured on MRS agar and after culturing for 48 h, separate white colonies on MRS agar were selected and

identified as *Lactobacillus spp.* based on their Gram-positive rod-shaped morphology, catalase and oxidase activity, sporulation, and cell motility. *Lactobacillus spp.* were the predominantly isolated organisms from the total bacterial isolates. The *Lactobacillus spp.* were further identified to the species level by using 16S ribosomal RNA sequencing analysis. A total of 13 *Lactobacillus* isolates, including *L. acidophilus* (n=6), *L. plantarum* (n = 3), *L. paracasei* (n = 2), *L. fermentum* (n = 1), *L. bugarius* (n = 1) were isolated in this study. In our experiments, *L. acidophilus* was considered since *L. acidophilus* was the most predominantly isolated organism. To ensure consistency and reproducibility, CFS was prepared from a single pure culture of *L. acidophilus* in all experiments.

Antimicrobial Susceptibility of Tested Pathogens

The antibiotics susceptibility testing of *K pneumoniae* and *P aeruginosa* isolates indicated that the tested strains were highly resistant to most tested antibiotics as ampicillin, amoxicillin, ciprofloxacin, trimethoprim/sulfamethoxazole, third-generation cephalosporins in a range of 66.6–100%. The antibiotics which showed good activity included imipenem, meropenem, gentamycin, and amikacin. All isolates were ESBL producers (Table 1).

Antibacterial Activity of CFS

CFS of lactobacilli exhibited antibacterial activity against all tested ESBL-producing strains but with variable degrees. The effect of CFS was greater on *K pneumoniae* than on *P aeruginosa*. The mean diameter of the inhibition zone was larger in case of *K pneumoniae* compared to *P aeruginosa* isolates (17±2.4 mm and 13±1.3 mm respectively; p < 0.05) as shown in Table 2. To confirm these results, we tested the effect of CFS on the viability of *K pneumoniae* and *P aeruginosa* using the MTT assay. CFS was able to reduce the viability of *K pneumoniae* and *P aeruginosa* strains by 65%±13 and 53%±15 respectively after only 6 h of incubation compared to control samples incubated in the absence of CFS.

Kinetics of Bacterial Growth in Lactobacilli CFS-Containing Media

Figure 1 shows the results of the time-killing test and assessment of the ability of CSF to suppress the growth of ESBL-producing *K pneumoniae* and *P aeruginosa*. The growth of *K pneumoniae* and *P aeruginosa* was significantly inhibited

Table 1 Antibiotic Resistant Pattern of *K pneumoniae* and *P aeruginosa* Isolates

Antibiotic	<i>K pneumoniae</i> n=15	<i>P aeruginosa</i> n=15
Ampicillin	15 (100%)	15 (100%)
Amoxicillin	15 (100%)	15 (100%)
Amoxicillin/clavulanic acid	15 (100%)	15 (100%)
Trimethoprim/sulphamethoxazole	15 (100%)	15 (100%)
Aztreonam	11 (73.3%)	10 (66.6%)
Cefepime	10 (66.6%)	15 (100%)
Cefotaxime	13 (86.6%)	13 (86.6%)
Cefoperazone	15 (100%)	14 (93.3%)
Ceftazidime	13 (86.6%)	15 (100%)
Ceftriaxone	13 (86.6%)	15 (100%)
Ciprofloxacin	15 (100%)	15 (100%)
Imipenem	2 (13.3%)	3 (20%)
Meropenem	0 (0%)	1 (6.6%)
Gentamicin	0 (0%)	0 (0%)
Amikacin	0 (0%)	0 (0%)
ESBL production	15 (100%)	15 (100%)

Table 2 Inhibition Zone Diameters Induced by CSF on *K pneumoniae* and *P aeruginosa*

Bacterial Strain	<i>K pneumoniae</i>	<i>P aeruginosa</i>
	Diameter (mm)	Diameter (mm)
Strain #01	19±2	13±1
Strain #02	17±2	13±2
Strain #03	12±2	13±1
Strain #04	18±1	14±1
Strain #05	16±1	15±2
Strain #06	15±1	16±1
Strain #07	16±3	12±1
Strain #08	17±1	13±2
Strain #09	14±1	14±2
Strain #10	15±2	15±2
Strain #11	15±1	12±1
Strain #12	18±1	12±1
Strain #13	19±1	11±3
Strain #14	21±1	13±1
Strain #15	20±1	14±1
Mean	17±2.4	13±1.3

after culturing with CFS in all tested time points compared to control cultures incubated without CFS (all $p < 0.05$ except *P aeruginosa* at 4 h). The inhibitory effect was more prominent on *K pneumoniae* cultures, as indicated by the lower CFU, particularly after 24 h of incubation. CFS induced about 2.1 log reduction in the growth of *K pneumoniae* and about 1.5 log reduction of *P aeruginosa* growth compared to control cultures incubated in the absence of CFS.

Assessment of the Antibiofilm Activity

All tested strains produced an $OD_{570} > 0.24$ and were classified as potent biofilm producers. A dose-dependent reduction in biofilm formation was observed when different concentrations of CFS were added to the growing biofilms of *K pneumoniae* and *P aeruginosa*. Moreover, when 24 h biofilms were challenged with CFS, the formed biofilms were disrupted and removed by $52\% \pm 12$ and $41\% \pm 15$, respectively (Figure 2).

Effect of CFS on Bacterial Invasion Ability

Virulent bacteria can invade target cells and initiate their damage. We, therefore, tested the invasiveness of *K pneumoniae* and *P aeruginosa* strains in the presence of CFS. Based on the flow cytometry histogram (Figure 3), FITC-labelled *K pneumoniae* and FITC-labelled *P aeruginosa* strains displayed a similar degree of invasiveness to the 293 cells. Interestingly, CFS was able to decrease the invasive ability of these pathogens. The mean percent of intracellular FITC-*K pneumoniae* was reduced from $30.3\% \pm 7$ to $15.4\% \pm 5$ (14.9% reduction of *K pneumoniae* invasion) while the mean percent of FITC-*P aeruginosa* decreased from $36.9\% \pm 7$ to $25.2\% \pm 5$ (11.7% reduction of *P aeruginosa* invasion).

Discussion

In this study, we described the inhibitory activities of lactobacilli's CFS on ESBL producing *K pneumoniae* and *P aeruginosa*. Lactobacilli are well-known probiotics that are commonly used in dairy products.²⁴ These probiotic strains exhibit antibacterial activities through different mechanisms, including the production of organic acids, hydrogen peroxide, antimicrobial peptides, and bacteriocins.^{25,26} CFS of *Lactobacillus Casei* had anti-Shigella activities in vitro. This antibacterial activity was attributed to the production of organic acids since neutralization of the supernatants abolished their antibacterial effects.²⁷ Similarly, lactobacillus CFS exhibited antibacterial activities against a wide range of pathogens, including *Listeria monocytogens*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus subtilis*, *Salmonella typhimurium* and *E coli O157:H7*, *S. typhimurium*, *E. cloacae*, *P. aeruginosa*, *E. faecalis* and *Clostridium difficile*, *H. pylori*, *Campylobacter jejuni*.^{26,28–31} CFS preparations for 46 lactic acid bacteria isolated from different raw and fermented milk products exhibited antibacterial activities against *Enterococcus faecalis*, *E. coli*, *Salmonella spp*, *Shigella sonnei*, *Staphylococcus*

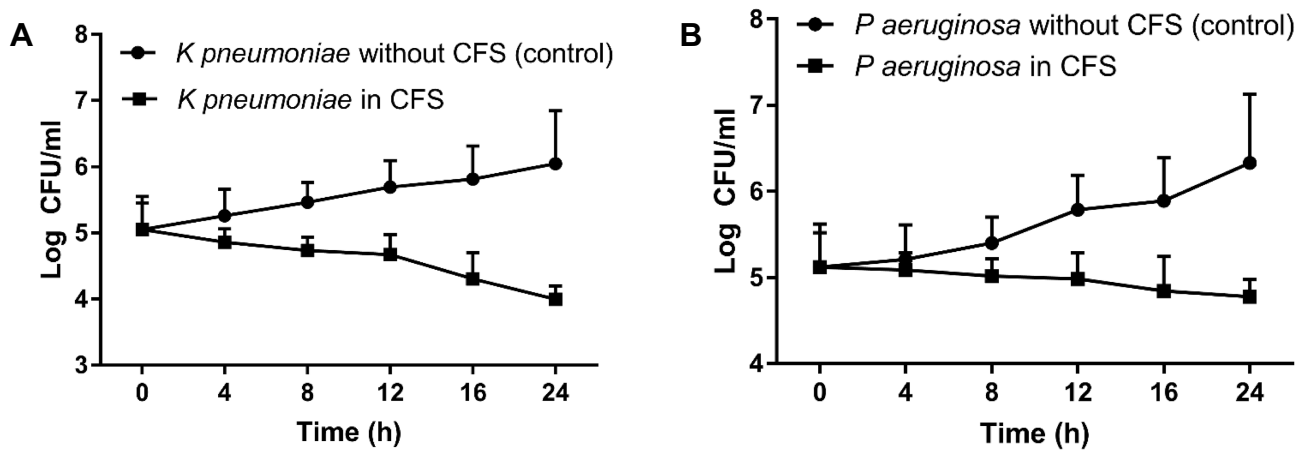


Figure 1 CFS of *L. acidophilus* decreased the growth of both *K. pneumoniae* (A) and *P. aeruginosa* (B) in a time-dependent manner.

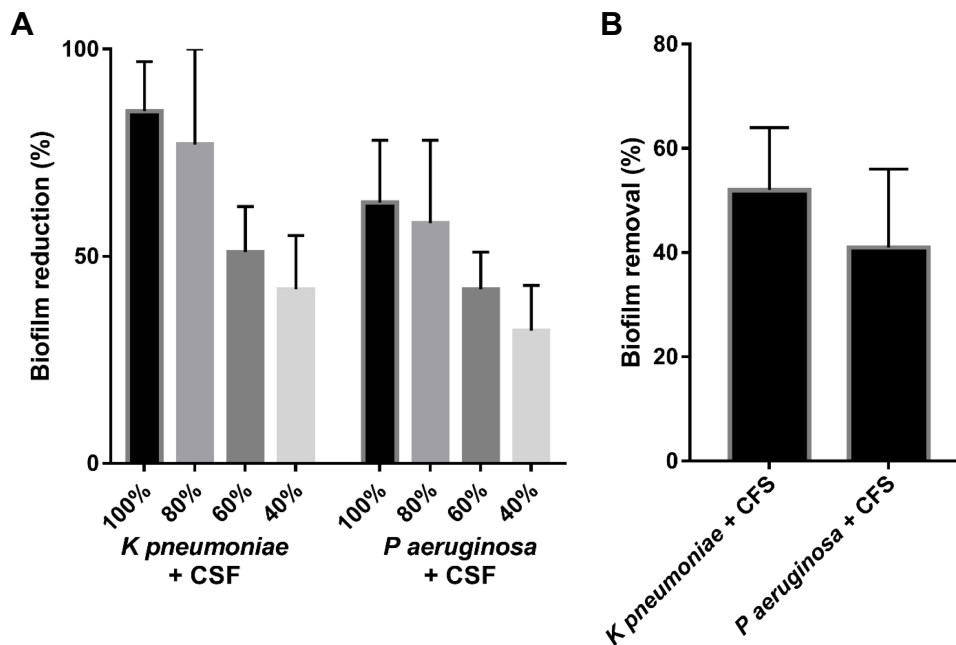


Figure 2 Anti-biofilm activity of different concentrations of *L. acidophilus*-CFS against *K. pneumoniae* and *P. aeruginosa* (A), and against 24 h-old biofilms (B) of *K. pneumoniae* and *P. aeruginosa*.

aureus, *MRSA*, and *Listeria monocytogenes*.³² Interestingly, the antimicrobial activity of probiotics is not confined to bacteria. Yasui, Kiyoshima, Ushijima³³ showed that probiotic administered provided protection against rotavirus-induced diarrhea. Moreover, it was demonstrated that the inhibitory activity of lactobacilli supernatants was not affected by environmental factors and storage conditions²⁹ which is an advantage if CFS is used commercially as a therapeutic agent.

In addition to their antimicrobial activities, consumption of lactic acid bacteria has many health benefits, including enhancement of the immune system, prevention of intestinal infections, increasing lactose metabolism, decreased levels of blood ammonia and cholesterol, and strong tolerance to gastric acid and bile.^{34,35} In addition, CFS of *Lactobacillus* spp. derived from Malaysian kefir had a strong antioxidant activities.³⁶ Some lactic acid bacteria have shown promising results in controlling the

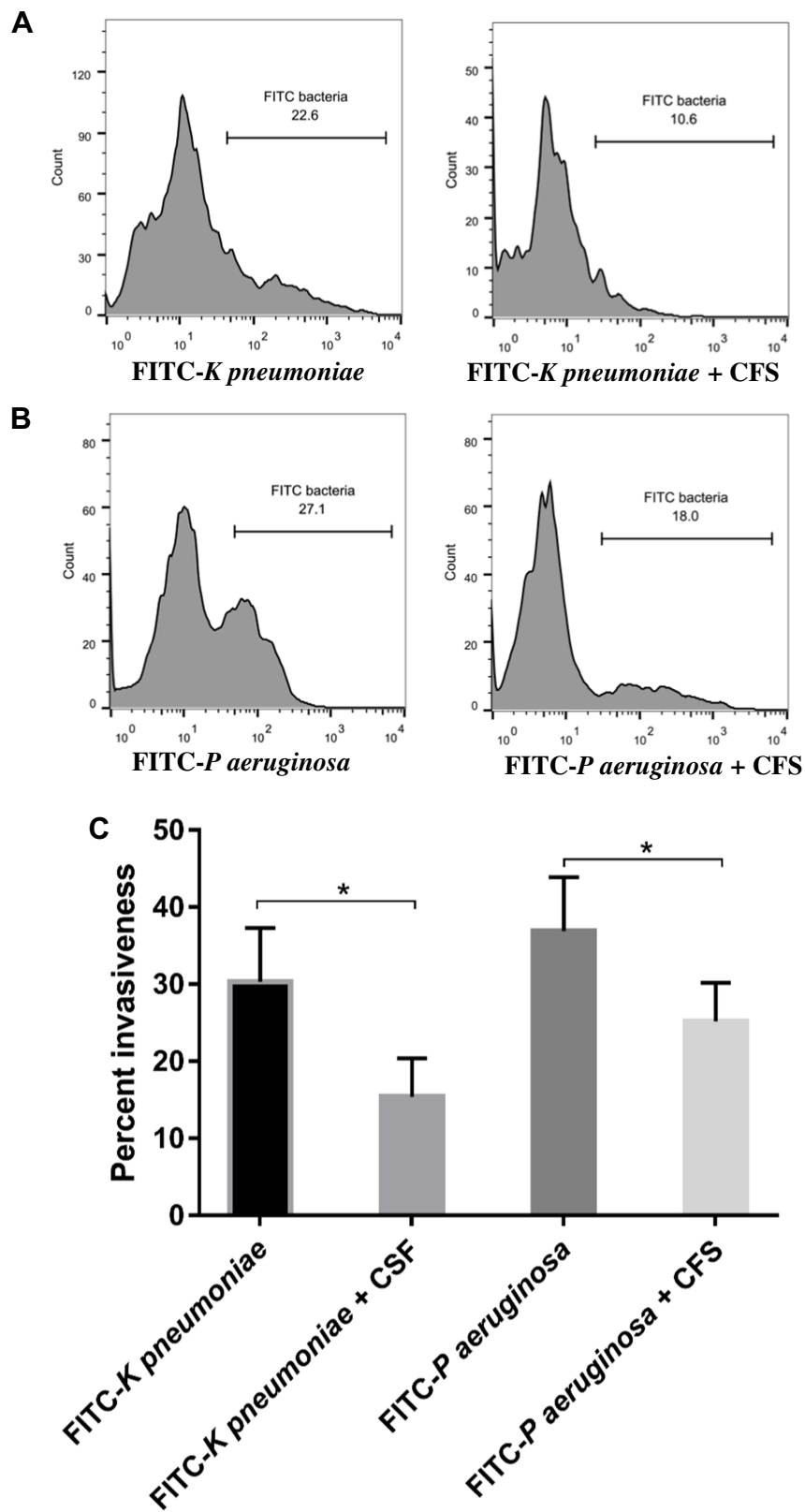


Figure 3 CFS of lactobacilli decreased the invasive ability of FITC-labelled *K pneumoniae* (A) and FITC-labelled *P aeruginosa* (B) on 293 cells. The mean percent of 15 independent experiments using 15 different clinical isolates of *K pneumoniae* and *P aeruginosa* is shown in (C). 293 Cells were treated with indicated FITC-labelled bacteria in normal culture or in the presence of CFS and analyzed after 6 h of incubation with flow cytometry. *Refer to significant difference with $p < 0.05$.

pathogenic bacteria in poultry, which in turn supports their use in improving the poultry performance and prevents transmission of poultry pathogens to humans.³⁷

We employed different strategies to confirm the inhibitory activity of the CFS. First, the classical well diffusion test was used as a screening tool. The ability of CFS to induce remarkable inhibition zones in all tested strains shows that the inhibitory activity is not strain-dependent. Given the fact that all tested *K pneumoniae* and *P aeruginosa* strains were ESBL producers, it could be concluded that CFS is an excellent strategy to get rid of these difficult-to-treat pathogens. Similar to our results, Chen et al¹⁰ showed that some *Lactobacillus* strains are able to inhibit carbapenem-resistant *Enterobacteriaceae*.

Different tests were used to explore the antimicrobial activities of lactobacilli's CFS. In the time-kill studies, the presence of CFS decreased the growth rate of both *K pneumoniae* and *P aeruginosa* by at least 1.5 logs after only 24 h. Additionally, CFS was able to reduce the viability of the strains, as evidenced by the MTT assay pointing to a decrease in the metabolic activity of these cells. Consistent with our results, *Lactobacillus acidophilus* isolated from the stool of an Iranian child induced a 90% reduction of *P aeruginosa* growth after 72 h of incubation.³⁸

Virulent bacteria are able to invade their target cells and initiate their damage. We, therefore, tested the invasiveness of *K pneumoniae* and *P aeruginosa* strains in the presence of CFS. According to our flow cytometry results, CFS induced a partial inhibition of the invasion of the Gram-negative pathogens. Our results provide another mechanism by which lactobacilli can prevent the pathogenesis of these bacteria. Previous reports have shown that lactobacilli have adhesive properties to the uroepithelial cells, which prevents colonization by uropathogens.^{39,40} Inhibition of the adherence and colonization of uropathogens to the uroepithelial cells is carried out by cell wall fragments of the lactobacilli, lipoteichoic acid, competitive exclusion, or steric hindrance.⁴¹ Healthy women who received encapsulated *Lactobacillus rhamnosus* GR-1 plus *Lactobacillus fermentum* RC-14 for 28 days, had correlated with a healthy vaginal flora.⁴² Taken together, the ability of probiotics to inhibit the adherence and invasive characters of uropathogens underlines their potential use in the prevention of genitourinary tract infections such as UTI or vaginitis.

Several experimental and clinical studies have assessed the potential use of lactobacilli for the prevention or treatment of infections caused by bacterial biofilms. CFS was able to reduce biofilm formation in both bacterial strains. The

antibiofilm activity of lactobacilli is due to their ability to inhibit the adherence of pathogenic bacteria to biological surfaces. The ability of CFS to inhibit and remove bacterial biofilms is linked to the presence of exopolysaccharides and bio-surfactants. Kim et al⁴³ reported that 1 mg/mL of exopolysaccharide of *L. acidophilus* could remove 87% and 94% of biofilms formed by enterohemorrhagic *E coli* on polystyrene and polyvinyl chloride microplates, respectively. *Lactobacillus* strain RC-14 produced a biosurfactant that has been reported to significantly inhibit the infection and adherence of *S. aureus* to surgical implants.⁴⁴

Generally, the CFS of the *L acidophilus* showed stronger antibacterial and antibiofilm activities against *K pneumoniae* than *P aeruginosa*. These results are in agreement with previously reported findings that a strain of *L acidophilus* isolated from a commercial vaginal product was not able to inhibit the growth of resistant clinical isolates of *P aeruginosa*. One mechanism proposed for the reduced anti-pseudomonas activity is the resistance of *P aeruginosa* to the antimicrobial substances in the CFS of the lactobacilli (hydrogen peroxide, lactic acid, and bacteriocin-like molecules).³⁸ *P aeruginosa* is intrinsically resistant to different antibiotics and can easily develop resistance to many other classes of antimicrobials such as those in the CFS of lactobacilli. Moreover, different virulence factors were observed in *P aeruginosa*, such as their elastolytic activity and dense biofilm formation, which may contribute to their ability to partially inhibit the effect of CFS.⁴⁵ *P. aeruginosa* has a low outer membrane permeability and expresses efflux pumps that can expel antimicrobial agents out of the cell leading to intrinsic resistance to these antimicrobial agents.⁴⁶ In addition to the intrinsic ability to resist antimicrobial agents, *P. aeruginosa* can gain resistance to antimicrobials through mutations or acquisition of resistance genes, which increases the challenges in the eradication of this pathogen and may lead to persistent infections.⁴⁷

For many years different investigators have elucidated the effective role of lactobacilli in the treatment of different pathological conditions. This work supports the therapeutic efficacy of lactobacilli in treating multi-drug resistant infections, particularly those caused by *K pneumoniae* and *P aeruginosa*, and elucidates some of the mechanisms by which CFS of lactobacilli can counteract these pathogens. Based on our results, we suggest the application of CFS of lactobacilli for controlling or preventing ESBL colonization or infection caused by the Gram-negative pathogens *K pneumoniae* and *P aeruginosa*.

Abbreviations

ESBL, extended-spectrum β -lactamase; CFS, cell-free supernatants.

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Disclosure

The authors report no conflicts of interest in this work.

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