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Investigation of the effect of the probiotic bacterium *Lactobacillus casei* on *oprD* gene expression in clinical isolates of imipenem-resistant *Pseudomonas aeruginosa*

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Abstract

OprD purine serve as a specific entry channel for drugs such as carbapenems in the outer membrane of *Pseudomonas aeruginosa*. Probiotics can play a role in inhibiting carbapenems resistance through efflux pumps by regulating the expression of OprD. This study aimed to investigate the effect of *Lactobacillus casei* on the expression of the *oprD* gene in clinical isolates of carbapenem-resistant *P. aeruginosa*. Bacteria were isolated and identified biochemically from clinical samples in Isfahan, and the carbapenem-resistant pattern was studied. The effect of *L. casei* and its cell-free culture supernatant (CFS) in De Man, Rogosa, and Sharpe (MRS) culture medium was investigated on *oprD* gene expression in the strains (one-way analysis of variance). A total of 37 *P. aeruginosa* strains were isolated, of which 54% were obtained from female **patients** and 46% from male patients. The highest number of strains (29.8%) was isolated from the age group of 21 to 30 years, and the lowest number (5.4%) was isolated from the age group of 51 to 60 years. The majority of strains were isolated from wounds (32.4%), and the lowest number was isolated from blood (19%). All strains produced biofilms, of which 10 (27%), 12 (32.4%), and 12 strains (32.4%) formed weak, moderate, and strong biofilms, respectively. The highest resistance was to the antibiotic imipenem (43.2%). All strains carrying the oprD gene exhibited resistance to the antimicrobial effects of the probiotic *L. casei* and its supernatant. However, the expression of the *oprD* gene was 67% and 36% of control after treatment with the probiotic and its CSF, respectively. Given the key role of the OprD porin in antibiotic-resistant strains of *P. aeruginosa*, inhibition of OprD production by *L. casei* and its supernatant is of particular preventive and therapeutic importance by controlling resistance to antibiotics in these strains.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Imipenem resistance, Probiotic, *Lactobacillus casei*, *oprD* gene expression.

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Introduction

Carbapenems are widely used antibacterial drugs for the treatment of bacterial infections. These drugs are able to cross the outer membrane of Gram-negative bacteria through the porin channels present in the outer membrane of bacteria and exert their effect by inhibiting peptidoglycan synthesis. However, resistance to carbapenems is usually caused by the production of beta-lactamase enzymes that are able to degrade the beta-lactam ring in the structure of these antibiotics. In addition, decreased permeability of the outer membrane and increased activity of efflux pumps can also reduce the effectiveness of these drugs. This resistance has complicated the treatment of carbapenem-resistant bacterial infections (1, 2). Multidrug efflux systems are composed of three protein complexes, including an energy-dependent pump in the plasma membrane, an outer membrane porin, and a binding protein. These systems remove toxic substances from bacterial cell. Efflux pumps transport molecules from the plasma membrane to the periplasm and then out of the cell. The interaction between the OprD porin and efflux pumps such as MexAB plays a key role in the development of antibiotic resistance. OprD-dependent resistance mechanisms include reduced expression of the *oprD* gene, mutations that create ineffective products or alter the function of the porin, gene promoter degradation, and the effect of metals such as zinc and copper on the reduction of *oprD* gene expression (3). Given the increasing drug resistance in *Pseudomonas aeruginosa*, finding compounds that can neutralize gene mutations and facilitate the entry of carbapenems through the OprD channel is of particular importance for the effective treatment of infections by the control of drug resistance (4).

Probiotics can improve the quality of life as an alternative to antibiotic treatments (5). *Lactobacillus casei* and its related species produce many bioactive metabolites that, when consumed, provide many benefits to the host. These benefits include improving digestive function, enhancing of the immune system, and antibacterial and antifungal effects. As such, many of their strains are considered probiotics because they can help maintain the microbial balance of the intestine and other parts of the body and prevent the growth of pathogenic bacteria. Additionally, these bacteria make the biological conditions unfavorable for the growth of pathogens by affecting the expression of genes essential for the pathogenicity of pathogens and preventing the formation of biofilms (6, 7). Considering the importance of the OprD porin channel in the intrinsic resistance of *P. aeruginosa* to antibiotics, the present study was conducted with the aim of detecting *oprD* gene in biofilm-forming and carbapenem-resistant isolates of *P. aeruginosa* and investigating the effect of *L. casei* and its culture supernatant on the expression of the gene in these isolates.

Materials and Methods

Sample collection and phenotypic identification tests

A total of 37 isolates of *P. aeruginosa* were collected from 100 clinical samples from different departments of Al-Zahra hospital in Isfahan, Iran. The samples included urine, blood, respiratory tract secretions, cerebrospinal fluid, abdominal fluid, and pericardial fluid. The samples were first cultured on Blood Agar (BA, HiMedia, India), and after 24 h of incubation at 37 °C, they were subcultured onto Trypticase Soy Agar (TSA, HiMedia, India) medium. After ensuring the purity of the colonies, Gram staining and biochemical tests were performed on them (8).

Determination of antibiotic susceptibility pattern by disk diffusion method

The Kirby-Bauer disk diffusion method was performed according to the Clinical and Laboratory Standard Institute 2024 (CLSI 2024) guidelines. First, pure colonies of *P. aeruginosa* were suspended in Phosphate Buffer Saline (PBS, HiMedia, India) to achieve a turbidity equivalent to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). The bacterial suspension was then swabbed onto the surface of Mueller Hinton Agar (MHA, HiMedia, India). Antibiotic disks—including Imipenem (IMP, 10 µg/disk), Cefepime (FEP, 30 µg/disk), Ciprofloxacin (CPFX, 10 µg/disk), Piperacillin/Tazobactam (TZP, 10 µg/disk), and Ceftazidime (CAZ, 10 µg/disk —were placed on the agar surface at a distance of 24 mm from each other and 14 mm from the edge of the plate. The plates were incubated at 37 °C for 24 h, after which the diameter of the inhibition zones were measured. According to CLSI 2024 guidelines, the strains were divided into 3 groups of resistant, susceptible, and semi-susceptible based on the diameter of the growth inhibition zones.

Biofilm adhesion analysis

The adhesion ability of biofilms was assessed using microtiter plates. Bacteria were cultured for 24 h in Trypticase Soy Broth (TSB, HiMedia, India) supplemented with 2% glucose. After adjusting the culture turbidity to 0.5 McFarland, 100 µL of bacterial suspension and 100 µL of TSB were added to each well of the microtiter plate. Negative control

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wells contained 200 µL TSB alone. The microtiter plate was then incubated at 37 °C for 24 h. Afterwards, the supernatant from each well was discarded, and the wells were washed three times using 300 µL PBS. Between each step, the plate was gently shaken to remove planktonic cells. The microtiter plate was then placed upside down on a paper towel to dry completely. After drying, 200 µL of 99% ethanol (Merck, Germany) was added to each well, and the plate was placed in a fixed location for 20 min to stabilize the biofilms formed near the ethanol. The contents of each well were then discarded, and the plate was kept at room temperature for 24 h to dry completely. After this period, 200 µL of 2% crystal violet (Merck, Germany) was added to each well, and the plate was placed in a fixed location for 15 min. The microtiter plate was then thoroughly washed with municipal water. At this stage, the formation of the biofilm was qualitatively visible based on color intensity. To fix the biofilms, 200 µL of 33% glycolic acid (Merck, Germany) was added to each well, and the optical density (OD) was measured using an ELISA reader (LabX, USA) at wavelength of 492-630 nm. The results were compared to the negative control. The OD cutoff (ODc) was determined as the blank OD. The experiment was performed in 3 replicates. Finally, the biofilm formation ability of bacterial strains was calculated based on the mean OD and ODc, as shown in Table 1 (9).

Table 1: Classification of bacterial biofilm formation ability based on optical density (OD) of microplate wells

OD mean	Biofilm formation
OD≤ODc	Non-adherent
ODc<OD≤ 2OD	Weak
2ODc<OD≤4ODc	Moderate
4ODc<OD	Strong

Investigation of the presence of *oprD* gene in biofilm forming strains

The presence of the *oprD* gene was evaluated in 16 strains of *P. aeruginosa* using PCR. For this purpose, a two-step DNA extraction kit (Cell Avand Pars, Iran) was used according to the manufacturer's instructions. The quality of the extracted DNA was assessed using electrophoresis on a 1% agarose gel in 1% TBE buffer. Primers were used to amplify a specific sequence in 16S rRNA gene in *P. aeruginosa*. The sequences of the primers are presented in Table 2. PCR reactions were performed using a reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer, 1-unit Taq DNA polymerase, and 0.5 µg/ml of template DNA in a total volume of 25 µl in a thermocycler device (Eppendorf, Germany). The reaction protocol included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing of primers at 59.3 °C for 60 s, and extension at 72 °C for 60 s. The reaction was terminated by a final extension at 72 °C for 5 min. Finally, the PCR products were visualized using 1% agarose gel electrophoresis.

Table 2: Specific primer sequences utilized for amplification of *oprD* gene (10)

Primer name	Sequence	Length (base)	Tm
Oprd F	5′-ATCTACCGCACAAACGATGAAGG -3′	23	60.5
Oprd R	5′-GCCGAAGCCGATATAATCAAACG-3′	23	60.5

Investigation the antibacterial effect of probiotic bacteria

The probiotic bacterium *L. casei* (ATCC 393) was obtained from the Iranian Research Organization for Science and Technology (IROST) and cultured in MRS Broth (Merck, Germany) at 37 °C for 24 h to reach a turbidity equivalent to 0.5 McFarland standard. Then, a suspension with a turbidity equivalent to 0.5 McFarland was prepared and centrifuged at 4000 g for 10 min. The supernatant was collected using a membrane filter with a pore diameter of 0.22 nm. The cell-free supernatant (CFS) and cell pellet were used separately to investigate their antibacterial activity and evaluate their effect on gene expression.

Investigation the antibacterial effect by agar well diffusion method

Clinical strains of *P. aeruginosa* carrying the *oprD* gene were selected and then treated with probiotic bacteria and their CFS. A 24-h culture of the strains with a turbidity equivalent to 0.5 McFarland was prepared in sterile TSB medium and spread evenly on the surface of MHA using a sterile cotton swab. Then, 6 mm diameter wells were created on the

surface of the agar plate, and the depth of each well was sealed with 10 µL of molten MHA to prevent liquid leakage. The central well served as a negative control, and 100 µL of sterile MRS broth was added to it. In 2 adjacent wells, 100 µL of *L. casei* cultured cells in MRS broth with a turbidity equivalent to 0.5 McFarland and 100 µL of CSF were added, respectively. The experiment was performed in 3 replicates, and finally, the plates were incubated at 37 °C for 24 h. The presence of growth inhibition zones was visually observed.

Study of the effect of probiotic cells and CFS on *oprD* gene expression

The presence of the *oprD* gene was detected in 12 strains of *P. aeruginosa*. These strains were cultured in TSB medium and incubated at 37 °C for 24 h. Then, the culture's turbidity was adjusted to 0.5 McFarland standard. Then, 300 µL of CFS was added to the first group, and 300 µL of bacterial sediment (with a turbidity equivalent to 0.5 McFarland) was added to the second group. The samples were then incubated in a shaking incubator at 37 °C for 24 h. The experiment was performed in triplicate. Reverse transcription real-time PCR was used to study gene expression. Total RNA was extracted from the samples using the SambioTM column extraction kit (Sm008, China) and cDNA synthesis was performed using a SambioTM kit (Sam020c, China) according to the manufacturer's instructions. Specific primer pairs (table 2) were used for gene amplification, and the 16S rRNA gene was amplified as an internal control. To perform the reaction, a mixture containing 10 µl Taq2x master mix (SYBER ROX, Peeshgam Biotechnology, Iran), 0.4 µL of each primer, 2 µL of cDNA, and 7.2 µL of RANase-free distilled water was used for each sample, with a final volume of 20 µL per reaction. The reaction was performed in a StepOneTM device, utilizing an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. Melting curve analysis was performed in the temperature range of 62 °C to 95 °C. Then, the data were analyzed by calculating $2^{-\Delta\Delta Ct}$ values. One-way analysis of variance (ANOVA) was used for data analysis.

Results

Phenotypic and biochemical identification

The results of the tests are shown in Table 3.

Table 3: Results of phenotypic and biochemical tests for initial identification of *P. aeruginosa* isolates.

Test	Gram staining	Motility	Oxidase	Catalase	Indole production	H_2S production	Citrate utilization	TSI	Gelatin hydrolysis	OF
Result	+	+	+	+	-	-	+	K/K Gas-	+	Oxidative

Distribution of the experimental samples

In this study, 37 strains of *P. aeruginosa* were isolated, of which 20 strains (54%) were from female patients and 17 strains (46%) were from male patients. According to these findings, the frequency of strains in women was 8% higher than in men. Out of 37 strains, the highest frequency was observed in the age group of 21 to 30 years (29.8%) and the lowest frequency was observed in the age group of 51 to 60 years (5.4%) (Fig. 1). Additionally, the distribution based on the type of sample showed that the highest were isolated from burn wound (32.4%) and the lowest were isolated from blood (19%) (Fig. 2).

Biofilm-forming ability of *P. aeruginosa* strains

Out of 37 *P. aeruginosa* strains, 10 strains (27%) formed weak biofilms, 12 strains (32.4%) formed moderate biofilms, 12 strains (32.4%) formed strong biofilms, and only 3 strains (8%) formed non-adherent biofilms.

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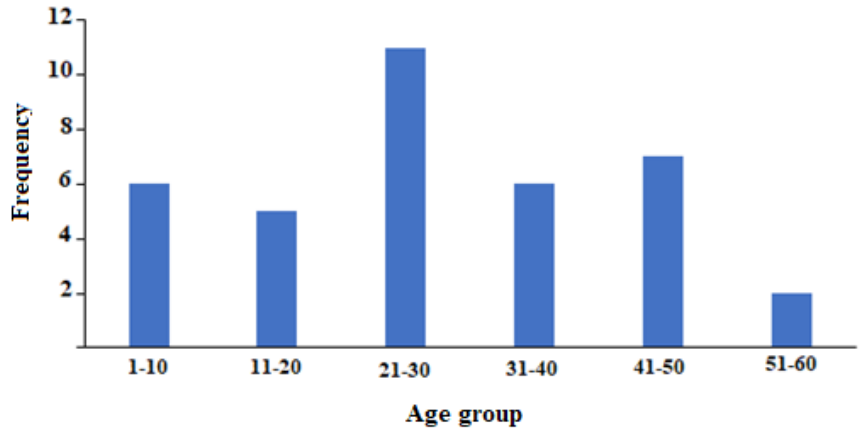

Fig 1: Distribution of *P. aeruginosa* strains among different experimental age groups.

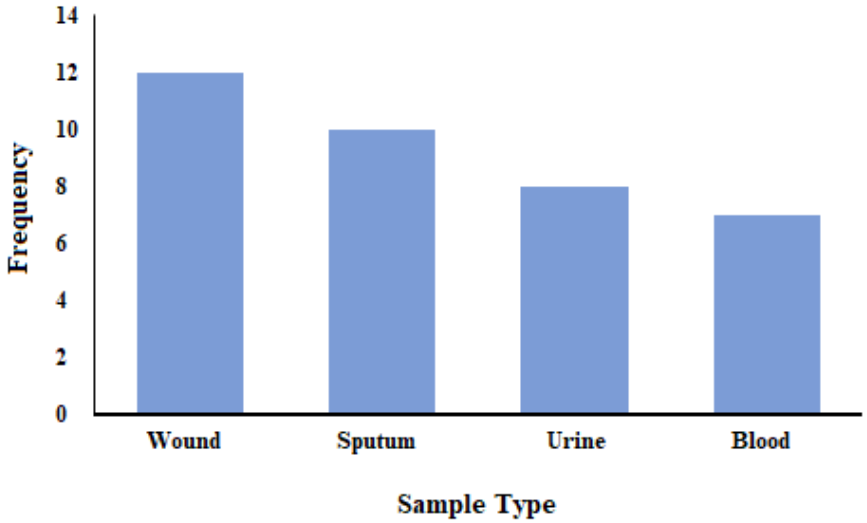

Fig 2: Distribution of *P. aeruginosa* strains among different experimental sample types.

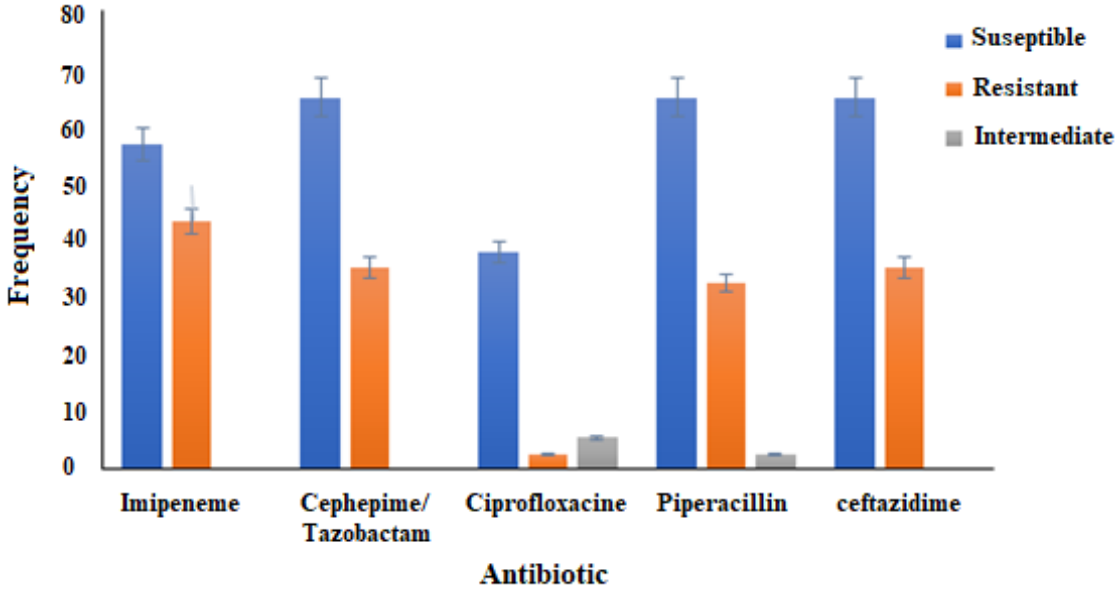

Fig. 3: Frequency distribution of antibiotic susceptibility among isolated strains of *P. aeruginosa*.

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Antibiotic susceptibility of strains

P. aeruginosa strains showed the highest susceptibility to the antibiotics cefepime, piperacillin/tazobactam, and ceftazidime (64.8%) and the highest resistance to the imipenem (43.2%) (Fig. 3). Imipenem-resistant strains (16 strains) were used for further investigation.

Frequency of the *oprD* carrying gene in imipenem-resistant and biofilm-producing strains of *P. aeruginosa*

Among 16 imipenem-resistant strains, 12 strains (75%) had *oprD* gene. The results of agarose gel electrophoresis of the PCR products are shown in Fig. 4.

Effect of the probiotic bacterium *L. casei* on imipenem-resistant strains of *P. aeruginosa*

All 12 imipenem-resistant strains of *P. aeruginosa* that carried the *oprD* gene showed resistance to the antibacterial effects of probiotic bacterium *L. casei* and its supernatant. A sample of well diffusion experiment results is shown in Fig. 5.

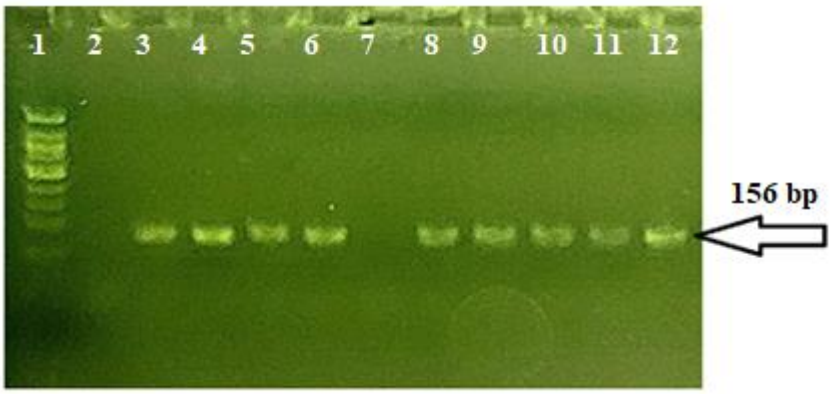

Fig. 4: Agarose gel electrophoresis of *oprD* PCR product. lane 1: 100 bp size marker, lane 2: negative control, lanes 3-6 and 8-12 imipenem-resistant strains of *P. aeruginosa* carrying *oprD* gene, and lane 7 a bacterial sample lacking *oprD* gene.

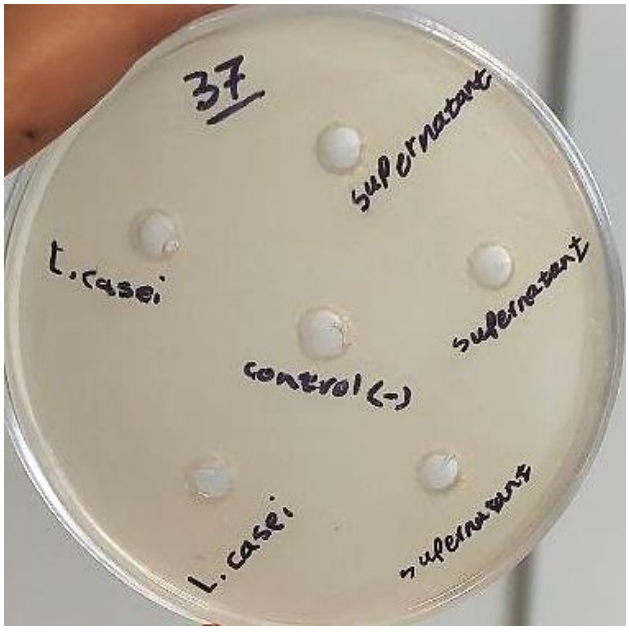

Fig. 5: Results of agar well diffusion method for investigation of the antibacterial effect of *L. casei* and its supernatant on an imipenem-resistant strain of *P. aeruginosa* carrying *oprD* gene. No growth inhibition zone was seen.

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Effect of probiotic bacterium *L. casei* on *oprD* gene expression in imipenem-resistant strains of *P. aeruginosa*

The expression of the *oprD* gene in strains treated with probiotic *L. casei* and its supernatant was quantitatively investigated using melting curve analysis and amplification curves (Fig. 6). As shown in the melting curves, the primers are specific for the gene under study, and no non-specific peaks, including primer dimers, are seen. To compare the expression of the *oprD* gene under treatment with probiotic and supernatant compared to the control, a one-way ANOVA test was performed. According to the test results, the expression of the gene under the influence of two different treatments differs at the 95% confidence level and is lower than the control. This reduction was 67% due to treatment with probiotics and 36% due to treatment with probiotic supernatant (Table 4).

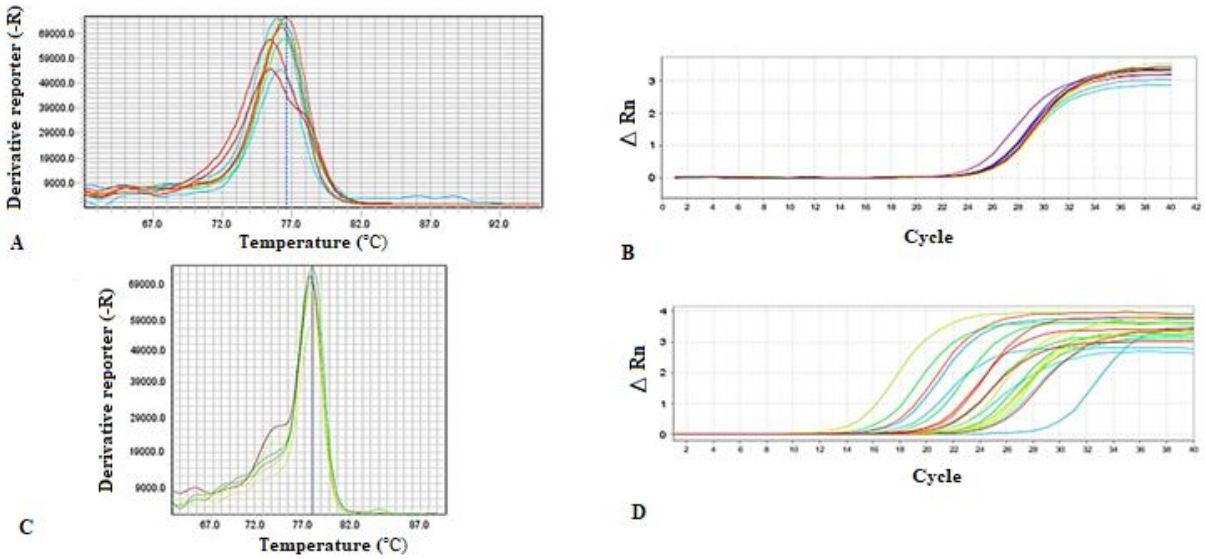

Fig. 6: Melting curve (a) and amplification curve (b) of the 16S rRNA gene and melting curve (c) and amplification curve (d) of the *oprD* gene in *P. aeruginosa* treated with the probiotic bacterium *L. casei* and its supernatant.

Table 4: Mean, standard deviation, and one-way ANOVA test results in investigating the effect of different treatments of *L. casei* and its supernatant on the expression of *oprD* gene in *P. aeruginosa*.

Treatment	Results	Confidence interval for the mean		ANOVA	
	ΔRn mean ± S.E	Lower limit	Upper limit	F	**Sig.**
Probiotic	0.327 ± 0.075 [c]	0.161	0.492	26.345	**0.000**
Supernatant	0.640 ± 0.085 [b]	0.852	0.452		
Control	1 ± 0.000 [a]	1	1		

Similar letters indicate the same mean values (α=0.05)

Discussion

In the present study, 37 strains of *P. aeruginosa* were isolated and identified from clinical samples in Isfahan. Of these, 54% were obtained from female patients and 46% from male patients, indicating a slightly higher distribution in women than in men. Dori et al. (11) observed that the frequency of *P. aeruginosa* isolates in Fars was higher in men (65%) and lower in women (35%). In contrast, Baghbani-Arani et al. (12) reported frequencies of 55% in men and 45% in women. Tran et al. (13) reported a frequency of 55.6% in men and 44.4% in women in southern Vietnam. Although studies indicate a slightly higher prevalence in men, this bacterium is capable of causing serious infections in various body systems in both sexes, especially in cases of weakened immune systems. A study has shown that estrogen in women can facilitate the transformation of *P. aeruginosa* from non-mucosal to mucosal forms, which exhibit higher virulence (14). Therefore, infection in women can be more significant in this regard. The results of the distribution of total strains among different age groups showed that, out of 37 strains examined, the

highest number of strains was isolated from the age group of 21-30 years (29.8%) and the lowest was isolated from the age group of 51-60 years (5.4%). The study by Al Hasan et al. (15) in patients with monomicrobial bacteremia caused by *P. aeruginosa* reported a mean age of 69 years. Notably, 78.4% of cases were either hospital- or healthcare-associated. Furthermore, most patients had multiple underlying diseases, which likely contributed to the increased frequency of infections in older adults. In the study of Tran et al. (13), the highest number of strains (84.4%) was obtained from the age group of 18-59 year–old-age group, and the lowest number of strains was obtained from the age group of over 60 years, which is similar to the results of the present study. The results of the present study showed that out of 37 strains of *P. aeruginosa*, the highest number of strains was isolated from burn wound (32.4%) and the lowest number was isolated from blood (19%). In the study by Dori et al. (11), the highest number of isolates (65%) was obtained from burn wounds, and the lowest number was isolated from eye samples.

The results of the evaluation of the antibiotic resistance pattern for *P. aeruginosa* strains isolated in the present study showed that the highest resistance was to the antibiotic imipenem (43.2%). The results of the study by Abdelraheem et al. (16) conducted on 97 clinical isolates of *P. aeruginosa* from wound and urine samples showed, that 58.8% of them were resistant to imipenem. This rate was reported as 65% in the study by Ahmad Hasan et al. (17) conducted on 20 isolates from burn infections in Najaf province, Iraq. Additionally, 85% of the strains were resistant to meropenem. The results of the present study and the aforementioned studies indicate the concerning frequencies of carbapenem resistant *P. aeruginosa* strains. Therefore, continuous monitoring of the antibiotic resistance pattern and the use of appropriate alternatives methods to antibiotics seem necessary.

In the present study, most of the imipenem-resistant strains of *P. aeruginosa* (75%) had the *oprD* gene. In a study by Ahmad Hasan et al. (17), which investigated the role of the *oprD* gene in carbapenem resistance in *P. aeruginosa* isolated from burn infection, all of the 20 carbapenem-resistant strains were reported to possess this gene. Given the role of Oprd in membrane permeability, a more detailed examination of the frequency, mutations, deletions, reductions, or increases in the expression of its coding gene, as well as its relationship with the potency and intensity of biofilm formation, can provide a clearer view of the resistance mechanism. On the other hand, understanding this relationship can help design more effective treatment strategies, especially in the selection of antibiotic combinations or the use of antibiofilm compounds.
All 12 imipenem-resistant strains of *P. aeruginosa* that had the *oprD* gene showed resistance to the antimicrobial effects of the probiotic *L. casei* and its supernatant, with no growth inhibition observed. Although the expression of *oprD* gene was significantly reduced by 67% and 36%, respectively, after treatment with the *Lactobacillus casei* and its culture supernatant. Mehboudi et al. (18) also evaluated the effect of probiotic culture supernatant on the expression of the resistance genes *mexD*, *mexB*, *mexF*, *ampC*, and *oprD*. The supernatant inhibited the growth of the isolates to some extent, although the effect was not significant. Meanwhile, the supernatant reduced the expression level of resistance genes. Interestingly, an increase in *oprD* expression was also observed in some groups. A study by Mishra and Singh (19) focused on the CzcR and CopR proteins, which are part of the two-component signal transduction systems in *P. aeruginosa*. Both proteins negatively regulate the expression of the OprD porin, which affects the uptake of antibiotics such as carbapenems. Molecular docking was performed on these proteins to identify potential inhibitors. Efficient inhibitory ligands were evaluated based on their minimum binding energy and human oral absorption. The resulting ligands showed highly potent inhibitory properties and satisfactory pharmacokinetics compared to previously identified inhibitors of two-component signal transduction systems in Gram-negative bacteria. These potential inhibitors can be further evaluated in vitro to assess their efficacy inhibiting *P. aeruginosa* biofilms. The results of the present and other studies suggest that the mechanism of antibacterial action is most likely related to the regulation of efflux pumps. Efflux pumps play a key role in multidrug resistance by actively removing antibiotics from bacterial cells. These pumps utilize energy to extrude antibiotics from the cell, preventing them from reaching toxic concentrations and allowing the bacteria to survive. These pumps are regulated by their genes, which can be located on the bacterial chromosome or on mobile genetic fragments (plasmids), leading to innate or acquired resistance (20). In this context, identifying potential strategies may lead to the development of new therapeutic agents that disrupt *P. aeruginosa* biofilms.

Conclusion

The high biofilm formation potential and drug resistance observed in imipenem-resistant *P.*

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aeruginosa strains in the present study poses a public health risk. Given the key role of OprD porin in the antibiotic resistance of *P. aeruginosa*, the inhibition of its production by *L. casei* and its supernatant in the present study is of particular preventive and therapeutic importance in the control of imipenem-resistant *P. aeruginosa* strains.

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Conflict of Interest Statement

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