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Original Article

The Inhibitory Effects of *Lactobacillus Agilis* Against *Pseudomonas Aeruginosa* Biofilm Formation and Evaluation of *PpyR* (PA2663) and *algD* Gene Expression

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A B S T R A C T

Background and Aims: Hospital infections and their antibiotic resistance have become a global concern recently. One of the most prominent factors in hospital infections is *Pseudomonas aeruginosa (P. aeruginosa)*, which can become resistant to many antibiotics due to its ability to form biofilms. Recently, scientists have tried to replace antibiotic therapy with alternative therapies such as probiotics which can reduce or eliminate the pathogenic bacteria's ability to form biofilms. Therefore, the present study revealed that some genes, such as *algD* and *PpyR*, were involved in biofilm formation in *P. aeruginosa*. Furthermore, the inhibitory effect of the supernatant of *lactobacillus agilis* on the biofilm formation of *P. aeruginosa* was evaluated in the current study.

Materials and Methods: In this study, the effect of the supernatant of probiotic *Lactobacillus agilis* on the biofilm formation of *P. aeruginosa* and also the expression of two genes effective in biofilm formation (*algD* and *PpyR)* were investigated. Antibiograms were performed to detect the most resistant bacteria since there is a link between biofilm formation and antibiotic resistance. Further, the effects of probiotics on the expression of *PpyR* and *algD* genes were discussed.

Results: Results showed that the biofilm formation of *P. aeruginosa* was significantly reduced in the presence of *lactobacillus agilis*.

Conclusions: According to the current study, it could be concluded that because of antibiotics resistance and their associated mechanisms, probiotics could be used as a replacement for antibiotics in many treatments.

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Introduction

Recently, a serious problem has arisen from the resistance of pathogenic bacteria to antibiotics. *Pseudomonas aeruginosa (P. aeruginosa) is* one of the common causes of nosocomial and other opportunistic infections. Biofilm formation and antibiotic resistance are two major factors associated with developing long-term infections [1]. Biofilm is an extracellular polysaccharide that immobilizes the bacteria inside, protecting them from antimicrobials. Discontinuation of antibiotics causes the bacteria in the biofilm to grow and multiply, resulting in the return of an infectious disease [2, 3]. Biofilms act as a protective barrier and adhesion agent for bacteria [4-7]. The consistency of *P. aeruginosa* biofilm is related to different types of polysaccharides such as alginate, *psl*, and *pel*. Alginate is encoded by several genes, such as *algA, algU,* and *algD,* and *pel* and *psl* are encoded by various genes, such as *pelA, pelB, pslA, and pslB*. The *pel* operon is one of the essential components of the biofilm matrix in mucoid and non-mucoid strains. It initiates the adhesion of bacteria to the surface and intercellular communication and maintains the integrity and maturation of the biofilm. *pslA* encodes the exopolysaccharide involved in the biofilm structure [7-9].

PpyR is another gene involved in biofilm formation [10]. *PpyR* gene product increases biofilm formation by increasing the exopoly-saccharide derived from the *psl* operon [8, 9]. *PpyR* is a signal activator and regulator gene. Genetic production of *PpyR* increases the production of the Pyoverdin virulence factor, and the inactivation of *PpyR* suppresses 71 other genes involved in transcriptions [11]. *PpyR* is *psl* and Pyoverdin operon regulator [12]. It has been presumed that *PpyR* acts like a sensor in the cell membrane that regulates the production of exopolysaccharides and pyoverdin. Ghadaksaz et al. evaluated 104 *P. aeroginosa* clinical isolates and declared that 99% contained *PpyR* gene, confirming that *PpyR* has an important role in biofilm formation [13].

Likewise, alginate, produced in mucoid strains of *P. aeruginosa*, plays an important role in biofilm production. In *Pseudomonas*, alginate promotes adhesion and reduces bacterial particle capacity by reducing sugar nucleotide production by *algD*. Thus, it is crucial in chronic pulmonary infections [4]. It has been proven that alginate has a wide range of important functions, including biofilm maturation, bacteria protection against phagocytosis, opsonization, and reduction of antibiotic release in biofilms. The *algACD* operon controls alginate synthesis in *P. aeruginosa*. In addition to regulating alginate synthesis and transcription of *alg* genes, the *algD* gene is also responsible for the final production of GDP-mannuronic acid, an essential alginate component. Increased alginate production decreases lung function and survival chances, especially in cystic fibrosis patients. In mucoid strains, alginate can inhibit phagocytosis, produce antibiotic resistance, and form biofilms [1, 9, 14]. *AlgD* and *PpyR* play an essential role in biofilm formation. In addition to being 100% conserved, these genes are

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present in all biofilm-forming microbes. Therefore, it is very important to research these genes [8, 11]. New antibiotics and biofilm inhibitors have been developed as therapeutic strategies. Recent research suggests that probiotics are the most effective treatment for pathogenic biofilms. Probiotics have opened up new opportunities for fighting infectious biofilms. Compared to conventional antibiotics, probiotics cannot induce strong selective pressure on resistant isolates, and they are also less cytotoxic than quorum sensing suppressors. Probiotics are believed to stimulate the immune system and protect the host from pathogens. Probiotics inhibit pathogenic bacteria's activity through various mechanisms [15]. The findings of different studies indicate that *Lactobacillus Spp* can potentially reduce bacterial biofilm formation and treat a variety of infections, such as chronic constipation, ulcerative colitis, and inflammatory bowel disease as significantly reducing the chances of *clostridium difficile*-associated diarrhea [16-18]. Probiotics have gained more attention in recent years for their use in treating certain human diseases [19].

The co-culture of *Lactobacillus paracasei (L. paracasei) 28.4*, *L. fermentum 20.4*, and *L. rhamnosus* with *Candida albicans (C. albicans)* manifested antimicrobial activities against an opportunistic pathogenic yeast, thereby reducing its biofilm formation [15]. Furthermore, researchers found that *P. aeruginosa* and its associated antibiotic resistance have caused numerous problems. In the present study, *L. agilis* was used as a probiotic, and its effects on *P. aeruginosa* biofilm formation and the transcription of *algD* and *PpyR* were investigated phenotypically and genotypically.

Materials and Methods

The pathogenic bacteria were isolated from clinical specimens of patients at Gorgan hospital in Iran between September 2021 and August 2022. Microbiological and biochemical methods, such as pigment production in agar, oxidase test, and triple sugar iron (TSI) media (Merck, Darmstadt, Germany), were used to identify *P. aeruginosa* isolates. Bacteria were then grown at 42 °C [16]. To evaluate the susceptibility of the isolates to different antibiotics, disk diffusion was performed using Mueller-Hinton agar (Merck, Darmstadt, Germany) according to guidelines from the Clinical and Laboratory Standards Institute. There were seven antibiotic disks tested (MAST Diagnostics, Merseyside, UK): ceftazidime (CAZ, 30 µg), piperacillin/ tazobactam (PTZ, 100 µg/ 10 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), gentamicin (GM, 10 µg), amikacin (AK, 30 µg), tobramycin (TOB, 10 µg), imipenem (IMI, 10 µg), and meropenem (MEM, 10 µg). In susceptibility tests, *Escherichia coli* ATCC 25922 was used as a control. Multidrug-resistant *P. aeruginosa* (MDR-PA) refers to isolates resistant to more than one antimicrobial agent in three or more antimicrobial categories. The study was approved by the Research Ethical Committee of Tehran Medical Science, Islamic Azad University (IR.IAU.PS.REC.1400.325).

Besides, written informed consent was taken from all the participants, and all methods followed relevant guidelines and regulations.

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Biofilm formation

Biofilm formation was assessed quantitatively using colorimetric microtiter plate assay, as previously described by Stepanović et al. [17]. with some modifications. *P. aeruginosa* was cultured overnight and adjusted to the turbidity of a 0.5 McFarland standard. After diluting 1:100 in 200 µL tryptic soy broth (TSB) with 1% glucose (Merck, Darmstadt, Germany), the suspensions were transferred to sterile flat-bottomed 96-well polystyrene microplates. McFarland solutions (0.5, 1, and 3) were prepared from *L. agilis* and centrifuged, and the supernatant was separated and filtered by 0.2 µm filtration. Afterward, the filtered supernatant was added to the 96 well microplates (*P. aeruginosa*, and *P. aeruginosa* with *L. agilis* supernatant). Following 24 h of incubation at 37 °C, the wells were gently washed three times with sterile phosphate-buffered saline (PBS, pH 7.3). 99% methanol solution was used for 15 min to fix biofilms, and the solutions were removed. The plate was then air-dried, and 200 µL crystal violet 0.1% (Sigma Chemical Co., St Louis, MO, USA) was used to stain the biofilms for 5 min at room temperature, followed by rinsing with water and drying. The biofilm in each well was distained by 200 µL of 95% ethanol for 30 min. At 570 nm, the samples' optical density (OD) was measured on a microtiter plate reader (BioTek, Bad Friedrichshall, Germany). Experiments were run three times in triplicate.

Scanning electron microscopy

To conduct scanning electron microscopy (SEM), specimens were fixed in a glutaraldehyde solution of 2.5% at 4 °C for 2 h, washed three times with a PBS solution (1 min each), immersed in a 1% osmic acid solution at 4 °C for 2 h, and dehydrated serially in 50%, 70%, and 95% absolute ethanol solutions for 10 min. Risoamyl acetate was substituted for ethanol during dehydration for 20 min at 4 °C. The sample tissues were then dried in a vacuum, sprayed with an IB3 (IB5) ion-sputtering, and analyzed by SEM.

RNA extraction steps

RNX-plus kit (Sina Clone) was used to extract RNA. 1 ml ice-cold RNXTM–PLUS solution was added to 2 ml tube containing a homogenized sample and then vortexed for 5-10 secs and incubated at room temperature for 5 min. In the next step, 200 µl of chloroform was added and mixed well for 15 secs by shaking. It was then incubated on ice for 5 min, followed by centrifugation at 12000 rpm at 4 °C for 15 min. The Aqueous phase was transferred to a new RNase-free 1.5 ml tube, and an equal volume of isopropanol was added, mixed gently, and incubated on ice for 15 min. The mixture was centrifuged at 12000 rpm at 4 °C for 15 min. The supernatant was discarded, and 1 ml of 75% ethanol was added and vortexed shortly to dislodge the pellet and then centrifuged at 4 °C for 8 min. at 7500 rpm. The supernatant was discarded, and the pellet was dried at room temperature for a few minutes. Pellet was then dissolved in 50 µl of DEPC-treated water. To facilitate dissolving, the tube was placed in 55-60 °C water bath for 10 min.

cDNA synthesis

One microgram of extracted RNA was transferred to the microtube. The required

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materials, such as random primer, oligo dT, and reverse transcriptase enzymes, were added and left at 37 °C for 10 min and then at 85 °C for 5 secs (see Table 1). The reaction mixture's incubation occurred under specific conditions (see Table 2).

Real-time polymerase chain reaction (PCR)

RNA was extracted from treated and control samples. Takara Synthesis Kit was used to synthesize cDNA using the manufacturer's instructions after confirming quality with TRIzol reagent after 24 h. SYBR green method was used for real-time PCR, and *16srRNA* was used as a reference gene. The sequence of primers is listed in Table 3. A dye can be added to the PCR mixture, which creates a fluorescent signal by binding to the double-stranded DNA. This color is called SYBR green. This technique reports the total amount of double-stranded DNA present during PCR and at any time. During each real-time PCR cycle, the fluorescent signal increases as double-stranded DNA binds to SYBR green. Of course, the reported value may be higher than the actual value. The reason for this issue is the non-specific binding of primers to each other and the production of primer dimers, the production of non-specific products, and as a result, the amount of double-stranded DNA increases. The temperature program of this test started with 10 min at 95 °C for initial denaturation and then triple 30 secs with 95, 57, and 72 °C applied, respectively, for 35 cycles. In the end, the temperature of 72 °C was applied for the final extension for 10 min. The materials used and the qPCR program is listed in Table 4 and Table 5, respectively.

Statistical analyses

GraphPad Prism 7.0 was used to conduct the statistical analysis. The data in the SEM figures are the mean ± standard error of 3–7 replicate experiments. The statistical analysis was performed using a two-tailed Student's t-test or one-way ANOVA with Bonferroni's post-hoc test. A P-value less than 0.05 was considered significant. The results' accuracy was supported by a melting curve graph.

Table 1. Required materials for cDNA synthesis

Volume	Reagents
2 µl	5X PrimeScript buffer (for Real-Time)
0.5 µl	PrimeScript RT enzyme mix I
0.5 µl	Oligo dT primer (50 µM)*1
0.5 µl	Random 6 mers (100 µM)*1
500µg	Total RNA
variable	RNase free dH_2O
Total 10 µl	

Table 2. Temperature conditions for cDNA synthesis

37 °C 15 min	Reverse Transcription
85 °C 5 secs	Inactivation of reverse transcriptase by heat treatment
4 °C	

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Results

Gram staining: Staining revealed that the bacteria were Gram-negative, observed under a microscope as red basils (Fig.1).

Triple Sugar Iron Agar (TSI): This test confirmed that this bacterium is non-fermentative, and the I culture appeared as Alkaline/ Alkaline, H_2S negative, and had no gas production (Fig. 2).

Pigment production: *P. aeruginosa* can be seen in green to blue color in Moller's Hilton agar medium, which is colorless due to pyocyanin pigment (Fig. 3).

Oxidase test: Since these bacteria contain cytochrome oxidase enzymes, they are oxidase positive and oxidize the reduced reagent to make it purple (Fig. 4).

Antibiogram: The antibiogram showed that all 12 clinical strains were resistant to the following antibiotics: amikacin (AM), ciprofloxacin (CP), imipenem (IP), gentamicin (GM), tobramycin (TOB), and cefotaxime (CTX). Thus, no growth halo was observed around these antibiotics discs, or the observed diameter was less than the reported limit as a semi-sensitive or sensitive strain. Eleven strains were resistant to the antibiotic piperacillin (PIP), and ten were clinically resistant to ceftazidime (CZA).

Table 3. The sequence of primers used

Forward PpyR [28]	**Reverse PpyR**
5-CGTGATCGCCGCCTATTTCC -3	5-ACAGCAGACCTCCCAACCG -3
Forward *algD* [29]	**Reverse** *algD*
5-GCGACCTGGACCTGGGCT-3	5-TCCTCGATCAGCGGGATC-3
Forward *16srRNA* [30]	**Reverse** *16srRNA*
5'-GAGGAAGTTGGGGATGACGT-3'	5'-AGGCCCGGGACGTATTCAC-3'

Table 4. Used materials in RT PCR

Real-Time Master-Mix	10X
Forward primer	100µM
Reverse primer	100µM
cDNA	10-50ng/ml
Deuterium depleted water	Variable

Table 5. qPCR program

10 min	95° C (Initial Denaturation)
30 secs	95 °C
30 secs	57 °C
30 secs	72 °C
10 min	72 °C (Final extension)

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Among 50 strains evaluated, 12 had the highest antibiotic resistance to first-line drugs (Multi Drug Strains Resistance) and were evaluated for further study based on the direct relationship between biofilm formation and antibiotic resistance. Real-Time PCR further assessed these resistant strains (Fig. 6a).

Biofilm formation: OD measurements were taken at 620 wavelengths following the formation of biofilms and the proximity of probiotics to samples. Based on the biofilm diagram of *P. aeruginosa*, McFarland decreased significantly in all three concentrations of the 0.5, 1, and 3 samples in the presence of probiotic strains compared to the control samples ($p = 0.001$, 0.05, and 0.05, respectively). The decrease in biofilm production at 0.5 McFarland concentration, on the other hand, was greater than at two concentrations of 1 and 3 McFarland, both of which were statistically significant ($p= 0.001$). Both McFarland concentrations of 1 and 3 were reported to be equivalent due to the declining biofilm production process (Fig. 5).

Electronic microscope image: In contrast to the control strain (*P. aeruginosa* alone), the biofilm production of *P. aeruginosa* strain in the presence of probiotic bacteria decreased significantly (Fig. 7).

Real-Time PCR: Evaluation of *algD* gene expression: Treatment with 0.5 and 1 McFarland reduced the expression of *algD* gene significantly in comparison to the control group (without any treatment), as shown in Fig. 8a ($p< 0.05$). Three McFarland concentrations (0.5, 1, and 3) had no significant effect on *algD* gene expression compared to standard treatment. However, as shown in Figure 8b, the decrease in the expression of these genes treated by two concentrations of 0.5 and 1 was significantly different compared to the control. In addition, treatment with McFarland concentrations (0.5, 1, and 3) was evaluated in *algD* gene and the result showed that treatment with McFarland of 0.5 was statistically significant. However, this decrease was not significant for the McFarland concentration of 1 (Fig. 8b).

Evaluation of *PpyR* gene expression: *PpyR* gene expression was significantly reduced in the groups treated with McFarland concentrations of 0.5 and 1 compared to the standard group ($p < 0.001$). However, despite the significant decrease in two concentrations of 0.5 and 1 McFarland, no significant changes were observed in the McFarland concentration of 3. Additionally, the decrease in *PpyR* gene expression of McFarland 0.5, 1, and 3 was significant ($p <0.001$).

Evaluation and comparison of *algD* and *PpyR* genes: According to Fig. 8d, the expression level of *PpyR* gene in the groups treated with probiotics at 0.5 and 1 McFarland concentrations decreased significantly compared to *algD* gene ($p < 0.001$), indicating that probiotic treatment at these two concentrations (0.5 and 1) was effective for *PpyR* gene expression. In contrast, as shown in Fig. 8d, under probiotic treatment with the concentration of 3 McFarland, this decrease compared to other concentrations of 0.5 and 1 McFarland was significant. Fig. 8a also illustrates the impact of *L. agilis* probiotic treatment on both *algD* and *PpyR* genes.

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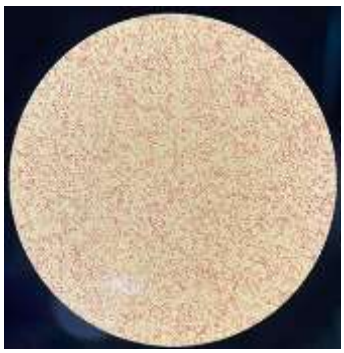

Fig. 1. gram staining result

Fig. 4. oxidase test result

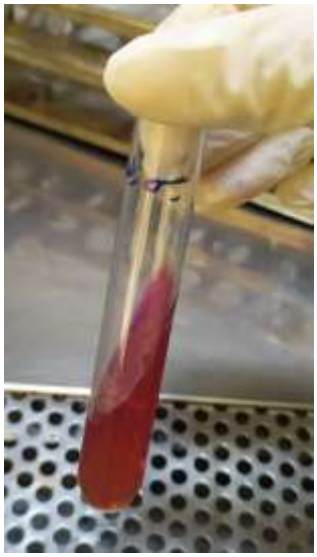

Fig. 2. TSI result

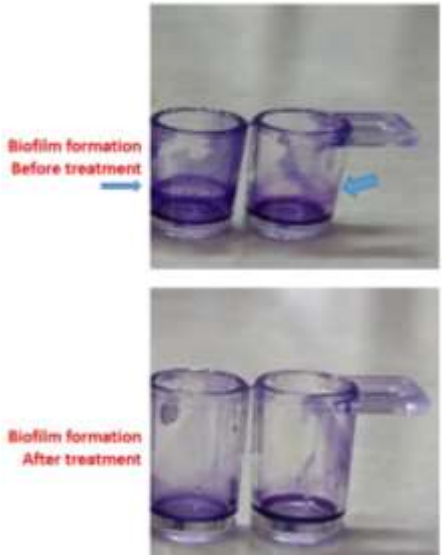

Fig. 5. *Pseudomonas aeruginosa* biofilm formation in presence of *Lactobacillus agilis* as a probiotic and without it

Fig. 3. pigment production result

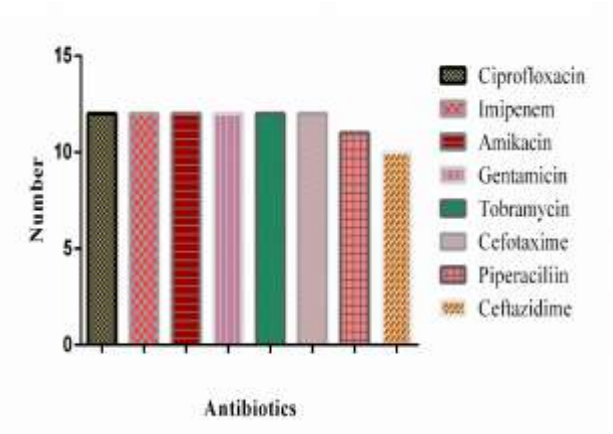

Fig. 6. Antibiogram results

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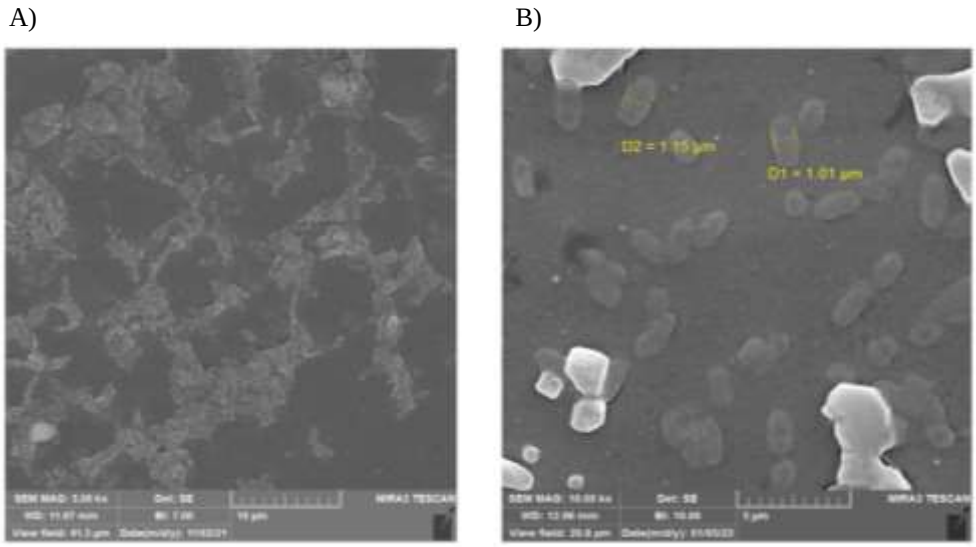

Fig. 7. A) *Pseudomonas aeruginosa* biofilm formation without probiotic treatment, B) *Pseudomonas aeruginosa* biofilm formation before along with *Lactobacillus agilis* as a probiotic

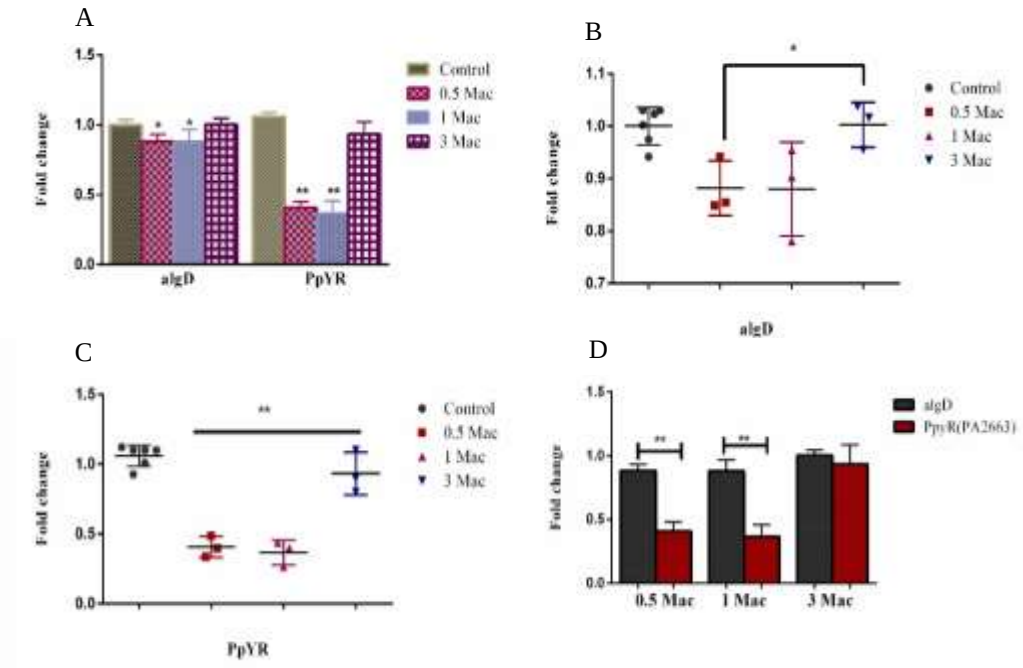

Fig. 8. A) Evaluation of the *algD* and *PpyR* genes expression at different concentrations. B) Evaluation of *algD* gene expression treated with three concentrations of probiotic bacteria. C) Evaluation of *PpyR* gene expression treated with three concentrations of probiotic bacteria. D) Evaluation and comparison of *algD* and *PpyR* gene expression with each other at different concentrations.

Overall, results showed that this probiotic had more effects on *PpyR* genes than *algD*. Biofilms and their infections are gaining attention due to the risks and side effects associated with high antibiotic doses and their high mortality rates.

Discussion

Researchers and physicians are constantly exploring new ways of treating or destroying biofilms. Probiotics, or non-pathogenic microorganisms, are among the most effective

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ways to remove biofilms. *Lactobacilli* have been approved as a probiotic for many years due to their high efficiency [18]. In the current study, the effect of *L. agilis* probiotic on the inhibition of biofilm formation of *P. aeruginosa* and the genes involved in biofilm formation (*algD* and *PpyR)* was investigated *L.agilis* probiotic was found to inhibit the growth of Enterotoxigenic *Escherichia coli 10* (ETEC10), reduce the expression of biofilm-producing related genes, and thereby diminishing its biofilm formation and mortality [19]. Another study found that *L. acidophilus* bacteriocin had antibiofilm activities against *P. aeroginosa* [20]. Evidence shows that supplementation with *L. fermentum* and *L.pelantarum* can benefit hospitalized patients and reduce the colonization of nosocomial multi-drug resistant bacterial strains such as *P. aeruginosa*, *Acinetobacter baumannii*, or *C. albicans* [21-23]. Furthermore, *L. casei* and *L.plantarum* isolated from traditional milk and yogurt inhibited *P. aeroginosa* biofilm formation. Herein, we demonstrated that *L.agilis* could significantly inhibit the biofilm formation in *P. aeroginosa*.

L. plantarum supernatant was shown to reduce the formation of *P. aeruginosa* biofilms [24]. Moreover, *L. fermentum* inhibited the growth of *P. aeruginosa* by preventing biofilm formation [25]. Another study reported that the co-culture of *L. acidophilus* and *P. aeruginosa* significantly reduced the growth of *P. aeruginosa*, and two other probiotics, *L. fermentum* and *L. plantarum*, had the least effect on *P .aeruginosa* growth. In addition, *Lactobacillus spp.* Moreover, their supernatants disrupted the biofilm formation of *P. aeruginosa* [26].

Researchers have speculated that *L. agilis* may also promote biofilm formation in *P. aeruginosa* based on previous research. Based on PCR results, the supernatant of *L. agilis* reduced *P. aeruginosa* at concentrations of 0, 1, and 3 McFarland.

However, the probiotic at the concentration of 0.5 McFarland showed the greatest effect on reducing biofilm formation. One study found that *Lactobacillus* strains reduced the formation of *S. mutans* biofilm, and *L. acidophilus* also reduced the expression of the *GtfB* and *LuxS* genes responsible for biofilm formation and maturation [27]. The current study showed that *L. agilis* reduced the expression of *algD* and *PpyR* genes that are highly responsible for biofilm formation by *P. aeruginosa*. The greatest reduction in the expression of these genes was associated with *P. aeruginosa* at 0.5 concentration of *L. agilis.* Therefore, reducing the highly conserved genes with a specific combination (like *L. agilis* as a probiotic) could be a promising treatment option.

Overall, it can be concluded that 0.5 McFarland probiotic is the best concentration for reducing biofilm production and expressing genes responsible for biofilm formation. Lower concentrations of probiotics may be more effective than higher concentrations since probiotic supernatants contain various substances and metabolites secreted by bacteria, and some of these substances may have opposite or different effects at higher concentrations. Further, several factors and genes effectively form *P. aeruginosa*

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biofilm, such as *algA, algU, algD, pslA,* and *PpyR.* Herein, we only examined two main genes (*algD* and *PpyR)*. This method can be used to study cell signaling pathways and the impact of the supernatant on intermediate genes and other pathway genes. A precise determination of the effective concentration can be made by analyzing the genes involved in the pathway.

Conclusion

Consequently, *L. agilis* at a concentration equivalent to half of McFarland can significantly reduce the production of biofilms by *P. aeruginosa* strains, and it can also significantly inhibit the expression of *algD* and *PpyR,* two genes crucial for biofilm formation. Future research in this area could help to treat *P. aeruginosa* infections.

Conflict of Interest

The authors declare that they have no conflicting interests.

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