Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Assessment of the anti-quorum sensing effect of *Lactobacillus* sp. metabolites on expression levels of QS-related genes in *Pseudomonas aeruginosa* PAO1

Pseudomonas aeruginosa PAO1'de QS ilişkili genlerin ekspresyon seviyeleri üzerine *Lactobacillus* sp. metabolitlerinin anti-quorum sensing etkilerinin belirlenmesi

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ABSTRACT

Objective: *Pseudomonas aeruginosa* is an important pathogen associated with nosocomial infections and its pathogenicity is mostly linked with the quorum sensing (QS) system. The aim of this study was to evaluate the anti-QS activity of the metabolites of vaginal *Lactobacillus* isolates and to investigate the effect of these metabolites on transcriptional regulation of QS related genes in *P. aeruginosa* PAO1.

Methods: In this study, 13 *Lactobacillus* isolates that were previously identified by 16S rRNA gene sequence analysis were used. Metabolites of these isolates were assessed for the anti-QS activity by using *Chromobacterium violaceum* CV12472. The influence of metabolites on the expression of QS related genes was also examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Results: All tested metabolites exhibited anti-QS activity with the appearance of a non-pigmented zone of *C. violaceum*. All tested quorum sensing-related genes (*lasI*, *lasR*, *rhlR* and *mvfR*) in *P. aeruginosa* PAO1 showed significant down-regulation after treating with the metabolites.

ÖZET

Amaç: *Pseudomonas aeruginosa* hastane enfeksiyonları ile ilişkili önemli bir patojen olup patojenitesi çoğunlukla quorum sensing (QS) sistemi ile ilişkilidir. Bu çalışmanın amacı, vajinal *Lactobacillus* izolatlarının metabolitlerinin anti-QS aktivitelerinin değerlendirilmesi ve metabolitlerin *P. aeruginosa* PAO1'in QS ilişkili genlerinin transkripsiyonel regülasyonu üzerindeki etkilerinin araştırılmasıdır.

Yöntem: Çalışmamızda daha önce 16S rRNA gen dizi analizi ile tanımlanmış olan 13 adet *Lactobacillus* izolatı kullanılmıştır. Bu izolatların metabolitlerinin, *Chromobacterium violaceum* CV12472 suşu kullanılarak anti-QS aktiviteleri değerlendirilmiştir. Metabolitlerin QS ile ilişkili genlerin ekspresyonları üzerindeki etkisi kantitatif revers transkriptaz polimeraz zincir reaksiyonu RT-qPZR ile araştırılmıştır.

Bulgular: Test edilen tüm metabolitler *C. violaceum*'un şeffaf zon bölgesi görünümü ile karakterize edilen anti-QS aktivite göstermişlerdir. Metabolitlerle temas sonrasında *P. aeruginosa* PAO1'de, test edilen tüm quorum sensing ilişkili genler (*lasI*, *lasR*, *rhlR* ve *mvfR*) anlamlı bir down-regülasyon göstermişlerdir.

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Geliş Tarihi / Received : 19.09.2019
Kabul Tarihi / Accepted : 03.12.2019

DOI ID : 10.5505/TurkHijyen.2019.57873

Kart D, Gürpınar SS, Eryılmaz M. Assessment of the anti-quorum sensing effect of *Lactobacillus* sp. metabolites on expression levels of QS-related genes in *Pseudomonas aeruginosa* PAO1. Turk Hij Den Biyol Derg, 2020; 77(3): 311-318

Conclusion: New anti-infection agents from natural resources with a different mode of action are necessary due to the increasing occurrence of antibacterial resistance. Our study highlights the possible usage of *Lactobacillus* metabolites as an anti-QS agent against *P. aeruginosa* biofilm cells in vitro.

Key Words: anti-quorum sensing activity, *Lactobacillus sp.*, metabolites, RT-qPCR. *Pseudomonas aeruginosa*

Sonuç: Antibakteriyel direncin artması nedeniyle farklı etki mekanizmasına sahip doğal kaynaklardan köken alan, enfeksiyonlara karşı yeni ajanlar gerekmektedir. Çalışmamız, in vitro *Pseudomonas* biyofilmlerinin kontrolünde *Lactobacillus* metabolitlerinin olası anti-QS ajanı olarak kullanılabileceklerini vurgulamaktadır.

Anahtar Kelimeler: anti-quorum sensing aktivitesi, *Lactobacillus sp.*, metabolitler, RT-qPCR, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is one of the significant opportunistic pathogens which causes numerous chronic infections such as severe burn infections, urinary tract infections, blood circulation and nosocomial infections (1). *P. aeruginosa* communicates and coordinates with other cells in a microbial population using small signaling molecules such as acyl-homoserine lactones (AHLs) known as quorum sensing (QS), contributing to its pathogenicity by regulating multiple virulence properties (2). QS system controls the expressions of various virulence genes depending on cell density and also triggers the biofilm formation which is one of the most important virulence factors of *P. aeruginosa* (3). *P. aeruginosa* has two QS systems known as *lasR/I and rhlI/R*, which use AHL signaling molecules. LasI is necessary for the synthesis of N- (3-oxododecanoyl) -L-homoserine lactone (3-oxo-C12-HSL) and lasR is a transcriptional regulator of virulence genes (4, 5). Among rhlI/rhlR synthase proteins, rhlI mediates the synthesis of the N-butyryl-homoserine lactone (c4-HSL) signaling molecule and RhlR is a transcriptional regulator (6). The synthesis of various virulence factors such as elastase, protease, exotoxin A, pyocyanin pigment production, rhamnolipids and biofilm formation which play an important role in cellular cytotoxicity and acute infections of *P. aeruginosa* are controlled by *lasR/I and rhlI/R* (7, 8).

QS inhibitors (QSIs) do not inhibit the growth of the pathogenic microorganism but lead to suppression of pathogenicity by decreasing virulence properties (9). Unlike conventional antibiotics targeting the cellular metabolic processes of the microorganism, QSIs inhibit the communication between the biofilm cells without exerting selective pressure for the development of resistance (3). Hence, inhibitor agents blocking the QS system in bacteria are considered as the possible options to fight with the infections caused by *P. aeruginosa*. The existence of bacterial persistence in chronic infections and increases in reduced susceptibility profiles to antimicrobials indicate the necessity of further investigations on anti-QS agents, especially from natural sources (5).

Lactobacillus species are normal flora members of mucosal surfaces in humans and some metabolites of *Lactobacillus* spp. such as lactic acid, acetic acid, hydrogen peroxide, and bacteriocin have the preservative property (10, 11). In the literature, the anti-biofilm effects of *Lactobacillus* spp. against *Staphylococcus aureus* and *P. aeruginosa* resistant strains were reported and many researchers have

focused on their use as alternative agents in the treatment of biofilm-associated infections (12-14).

The main objectives of this study were to investigate the anti-QS activity of the metabolites of vaginal *Lactobacillus* isolates and to evaluate the effect of these metabolites on the expression profile of QS-related genes in *P. aeruginosa* PAO1.

MATERIAL and METHOD

Lactobacillus isolates

In this study, metabolites of one *L. helveticus*, one *L. fermantum*, one *L. jensenii*, six *L. gasseri*, two *L. vajinalis*, and two *L. crispatus* vaginal isolates which were previously identified at the species-level by analyzing the 16S rRNA gene sequence were used (11).

Obtaining metabolites

A total of 13 *Lactobacillus* isolates were grown on Rogosa Agar for 24-48 hours following the inoculation into tubes containing 5mL of De Man-Rogosa Sharpe (MRS) Broth (pH 6.5) (Merck, Germany), under anaerobic conditions for 72 hours at 37°C. After the incubation period, the cells were removed by centrifugation at 12000 g, for 10 min, at 4°C. Cell-free supernatants (CFS) of the cells were filtered for the sterilization (0.45 µm pore size) (Minisart, Germany) (11, 15).

Biofilm formation

P. aeruginosa PAO1 was incubated for 24 hours at 37°C in Brain Heart Infusion (BHI) Broth. After the incubation period, final inoculum suspensions containing ~106 cfu/ml of P. aeruginosa were prepared in BHI. For each test condition, 100 µl of the inoculum suspensions were added to the wells of 96-well microtiter plates following the incubation for 4 hours at 37 °C. At the end of 4 hours, the wells were washed with phosphate buffer saline (PBS) three times in order to remove the non-adherent cells. After the washing step, the plates were incubated for an additional 20 hours to form mature biofilms (16).

Treating of the biofilm cells with the metabolites of *Lactobacillus* isolates

The metabolite of each isolate (100 µl) was transferred into the wells containing mature *P. aeruginosa* biofilms and the plates were incubated for 24 hours at 37 °C. After incubation time, the plates were vortexed for 5 minutes and then sonicated 3 times for 5 minutes. Biofilm cells were then transferred into a sterile tube and evaluated for anti-QS activity.

Detection of anti-QS activity

The agar well diffusion method was performed against reporter bacteria *Chromobacterium violaceum* ATCC 12472. One hundred microliters of the metabolites were loaded onto the wells (8 mm diameter) made on Luria Bertani Agar plates, pre-inoculated with *C. violaceum*. The plates were observed for the presence of zone of violacein inhibition after 24 hours of incubation at 30 °C (3). The appearance of clear zone of *C. violaceum* around the well loaded with the metabolites of *Lactobacillus* sp. isolates indicated the potential anti-QS activity.

Gene expression analysis

P. aeruginosa biofilms were grown and harvested and incubated with *Lactobacillus* sp. metabolites as described above. The mRNA expression changes of QS-related genes including *rhlR, rhlI, lasR, lasI* and *mvfR* in *P. aeruginosa* biofilms were assessed using quantitative polymerase chain reaction qPCR. Total RNA was extracted by RNA Isolation Kit according to the manufacturer's instructions (Roche Life Science). Total RNA was quantified in each sample using a NanoDrop spectrophotometer (BioDrop, Cambridge, UK). First-strand cDNA was synthesized by Evoscript Universal cDNA Master according to the manufacturer's instructions (Roche Life Science). To quantify cDNA, primers that correspond to *P. aeruginosa* genes were used (17). The sequences of the primers are presented

in Table 1. Real-time PCR (LightCycler 96 Instrument) was carried out with the Faststart Essential DNA Green Master (Roche Life Science) in 96-well plates in Roche LightCycler 96 Instrument (Roche, USA). Five µl of 1:2 diluted cDNA samples and 20 µl of master mix (containing the primers) were added to each well. The parameters for real-time PCR reactions included a single cycle of 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The formula, fold change = 2-ΔΔCt, was used to calculate the expression levels of tested genes (18). For the calculations of relative gene expression levels, these normalized data were used. Each test data from independent experiments were repeated at least three times.

Statistical analysis

Statistical data analysis was performed using the SPSS program (Version 23, SPSS, Chicago, IL, USA). All data are expressed as mean ± standard error. Student's t-test was used for comparisons between the *Lactobacillus* spp. metabolites treated and untreated groups. P values <0.05 were considered significant.

RESULTS

Anti-QS activity of *Lactobacillus* sp. metabolites

The QS inhibitory activity was determined by the presence of transparent inhibition zones around the wells that were filled with the metabolites. All tested metabolites showed anti-QS activity (indicated as A in Figure 1).

Additionally, the supernatants of *P. aeruginosa* cells were treated with the metabolites of *Lactobacillus* isolates in the biofilm environment which were examined for their anti-QS activity. Anti-QS effects of the metabolites of *Lactobacillus* isolates and the metabolites obtained after contact with *P. aeruginosa* biofilm cells were determined. Our results showed that there were decreases in the radius of inhibition zones of violacein pigment after exposure to the biofilm cells indicating that the biofilm environment leads to a decrease in the anti-QS activities of the *Lactobacillus* spp. metabolites (indicated as B in Figure 1).

Expression of QS genes in *P. aeruginosa*

The metabolites of isolates were tested on *P. aeruginosa* biofilms and their effects on the expression levels of genes encoding QS signal molecules that were responsible for the communications between the cells in biofilms were determined. The mRNA expression results of tested genes in *P. aeruginosa* were shown in Figure 2. Compared with the non-treated biofilm cells, mRNA levels of *rhlR, lasR, lasI* and *mvf* were significantly down-regulated in all treated biofilm cells of *P. aeruginosa* ($p<0.05$).

Table 1. Primers used in this study

Genes	Forward	Reverse
mvf	AACCTGGAAATCGACCTGTG	AACCTGGAAATCGACCTGTG
lasR	ACGCTCAAGTGGAAAATTGG	GTAGATGGACGGTTCCCAGA
lasI	CTACAGCCTGCAGAACGACA	ATCTGGGTCTTGGCATTGAG
rhlR	AGGAATGACGGAGGCTTTTT	CCCGTAGTTCTGCATCTGGT
proC*	GGCGTATTTCTTCCTGCTGA	CCTGCTCCACTAGTGCTTCG

*Housekeeping gene.

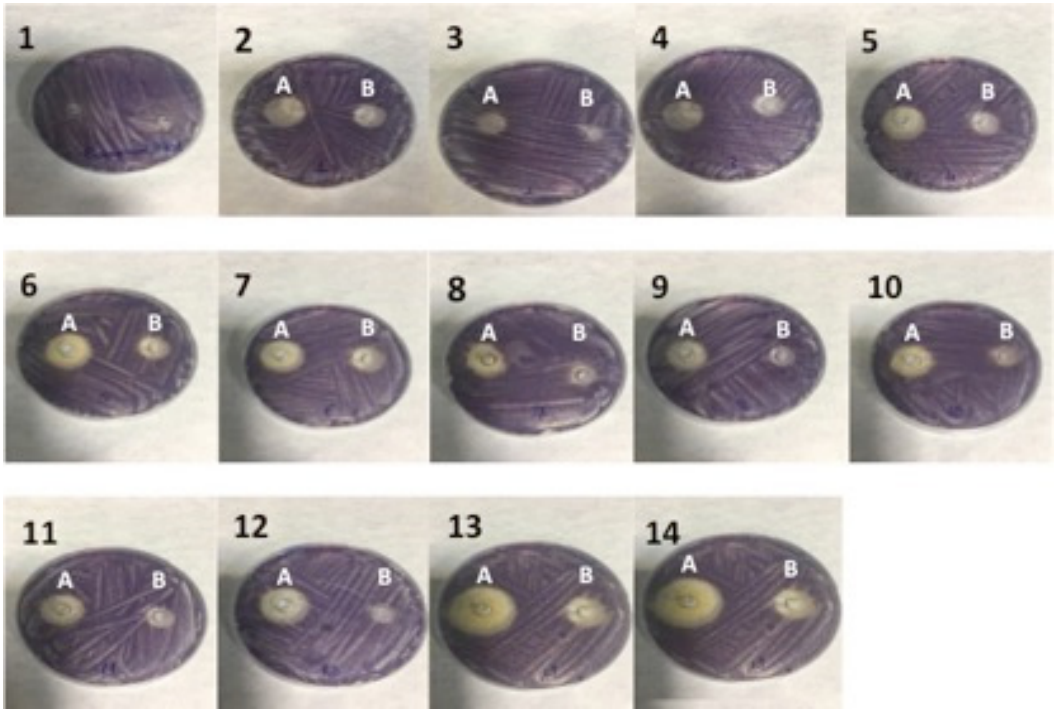

Figure 1. (A) QS inhibitory activity of the metabolites of *Lactobacillus* isolates (B) QS inhibitory activity of the *P. aeruginosa* biofilm supernatants treated with the metabolites of *Lactobacillus* isolates 1: MRS broth as negative control 2: *L. helveticus* 1; 3: *L. fermantum* 1; 4: *L. jensenii* 1; 5: *L. gasseri* 1; 6: *L. gasseri* 2; 7: *L. gasseri* 3; 8: *L. gasseri* 4; 9: *L. gasseri* 5; 10: *L. vajinalis* 1; 11: *L. crispatus* 1; 12: *L. vajinalis* 2; 13: *L. crispatus* 2; 14: *L. gasseri* 6.

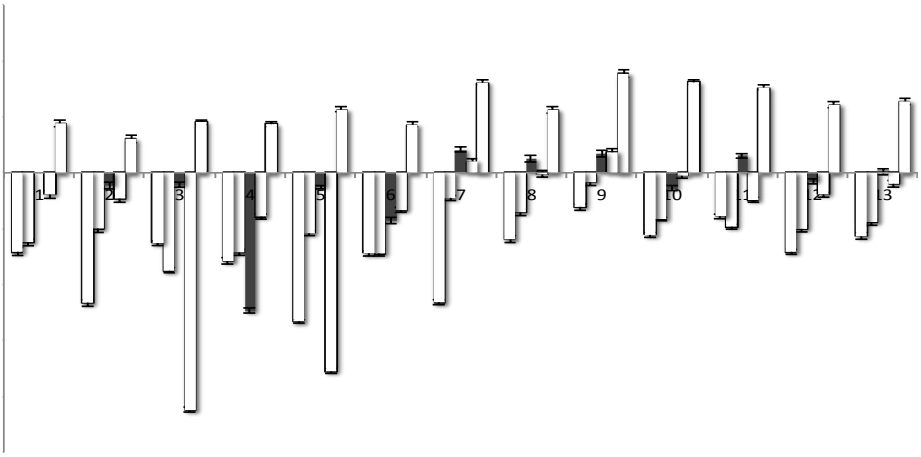

Figure 2. Relative expression results of QS-related genes of *P.aeruginosa* PAO1. P< 0.05 was considered statistically significant. 1: *L.helveticus* 1; 2: *L. fermantum* 1; 3: *L. jensenii* 1; 4: *L. gasseri* 1; 5: *L. gasseri* 2; 6: *L. gasseri* 3; 7: *L. gasseri* 4; 8: *L. gasseri* 5; 9: *L. vajinalis* 1; 10: *L. crispatus* 1; 11: *L. vajinalis* 2; 12: *L. crispatus* 2; 13: *L. gasseri* 6.

DISCUSSION

P. aeruginosa is one of the pathogens leading to chronic and persistent infections, usually caused by biofilms controlled by QS system (19). Current chemical agents are inadequate for the eradication of biofilms, hence new strategies that can be effective are needed. Results from several studies suggest that targeting the QS system in *P. aeruginosa* may be a new strategy to struggle against biofilm-related *P. aeruginosa* infections (20, 21). Activation of the QS system may lead to increases in virulence of pathogens such as *P. aeruginosa* and agents particularly inhibiting this system are called anti-QS inhibitors (22). The present study is particularly important in terms of investigating the anti-QS activity of bacterial metabolites of vaginal flora member *Lactobacillus* sp. which have attracted the attention of researchers in recent years. Our results show that suppression of QS-mediated violacein production of C. violaceum, when threated with the metabolites of 13 different *Lactobacillus* isolates, is a preliminary indicator that these strains have anti-QS property. The inhibition zones of violacein around the wells separately filled with both the metabolites of *Lactobacillus* isolates only and with the metabolites obtained after contact with *P. aeruginosa* biofilm cells were determined as anti-QS activity in both conditions. Our results suggest that the interaction with *P. aeruginosa* biofilm cells leads to decreases in the anti-QS activity of the isolates by a yet unknown mechanism.

In a study, antibiofilm effects of *L. pentosus* and *L. plantarum* metabolites isolated from fermented dairy products on *P. aeruginosa* and *Bacillus cereus* biofilms were reported (23). In another study, *L. rhamnosus* EMCC 1105 and L. gasseri EMCC 1930 strains were reported to be effective against *P. aeruginosa*, *Escherichia coli*, and *S. aureus* biofilms (24). Melo et al. showed that the metabolites of *L. fermentum* TCUESC01 and *L. plantarum* TCUESCO2 which were isolated from cocoa fermentation had antibiofilm effect on the biofilm of a resistant *S. aureus* CCMB 262 strain (13). In the study of Shokri et al., different metabolites such as lactic acid, acetic acid and formic acid produced by *L. fermentum* isolates showed an anti-biofilm effect on 80 *P. aeruginosa* isolates with multi-drug resistance. The usage of *L. fermentum* isolates as possible therapeutic agents in the control of resistant strains of *P. aeruginosa* has also been proposed in the same study (14).

Gene expression analysis results of anti-biofilm-specific agents have shown that they inhibit biofilm formation by causing a decrease in the expression of QS-related genes (17). The majority of virulence factors such as lasA-elastase, lasA-staphylolytic protease, toxA-exotoxin A, and aprA-alkaline protease produced by *P. aeruginosa* are synthesized through the rhl quorum sensing system that plays an important role in the irreversible attachment phase of the biofilm and the las system which positively controls the rhl system (25). In our study, we determined the transcription levels of QS-related genes such as *lasR/I*, *rhlR/I* and *mvfR* in order to evaluate the possible relationship between the inhibitory effect of *Lactobacillus* supernatants and the QS system. Significant reductions in mRNA levels of lasR/I, rhlR/I and mvfR genes were obtained for all tested *Lactobacillus* supernatants, which explains the anti-biofilm effect of these strains on *P. aeruginosa* biofilms that were observed in our previous study (12).

Soheili et al. have determined the effect of natural antimicrobial compound 3-Phenyllactic acid (PLA) produced by *Lactobacillus* spp. on biofilm formed by *P. aeruginosa* PAO1 and on QS system by using in vitro and in silico analysis. The results of the study showed that pyocyanin, hemolysin, protease, rhamnolipid and swarming activity were decreased and Rhl and Pqs related QS system was suppressed in the biofilm cells treated with PLA (26). Li et al. showed the bacteriocin-mediated antimicrobial effect of *L. plantarum* AB-1 and *L. casei* strains on

Shewanella baltica, the specific spoilage organism of the refrigerated shrimp. It was also concluded in the study that this antimicrobial effect was regulated by the AI-2 / LuxS-mediated QS system (27).

CONCLUSION

Many virulence genes may be overexpressed through the QS system in microorganisms following the transformation into more pathogenic forms. The discovery of new antimicrobial compounds targeting the QS system will result in inhibition of bacterial communication, thereby showing its activity without selective pressure on microorganisms. In our study, the metabolites of *Lactobacillus* vaginal isolates have been shown to have anti-QS activity. These natural compounds, alone or in combination with antibiotics, maybe future candidates for the treatment of *P. aeruginosa* biofilms in clinics.

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