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Original article

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Employment of Spore-Forming Probiotics to Combat Persister Cells of *Staphylococcus Epidermidis*

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Abstract

Background: In this study, spore-forming probiotics were employed to eradicate *Staphylococcus epidermidis* biofilms and the presence and expression of genes involved in stress response was examined**.**

Methods: Polymerase chain reaction (PCR) assay was used to detect *rpoS*, *relA* and *mazF* genes in *S. epidermidis* ATCC 12228. Biofilm production was investigated by microtiter plate (MTP) assay**.** 100X minimum inhibitory concentration (MIC) of gentamycin was used to induce persister cells in planktonic and biofilm bacterial cells. The expression of *rpoS*, *relA*, and *mazF* genes was assessed at different time intervals of 2, 8, and 24 h using real-time PCR assay. Then, dilutions of 1, 0.5, and 0.25 µg/ml of the supernatant of *Bacillus coagulans* culture was used to eradicate the persister cells and the number of colonies was determined.

Results: Persister cells of *S. epidermidis* were formed after 7 h in planktonic and 5 h in the biofilm structure after exposure to 50 µg/ml of gentamycin. The expression of *mazF* and *rpoS* in biofilm structure and the expression of *rpoS* and *relA* in persister cells were significantly higher compared to the control ($p < 0.05$). The number of persister cells showed a reduction of log 2.4 and log 0.8 after exposure to 1 and 0.5 µg/ml *B. coagulans* supernatant, respectively, but no reduction was observed at the concentration of 0.25 µg/ml.

Conclusions: The results showed that the supernatant of probiotics containing their secretive metabolites can be used as a novel approach to combat persister cells.

Keywords: Biofilm, Persister, Real-time PCR, Probiotics, *Staphylococcus epidermidis*.

Introduction

Staphylococcus epidermidis is a Gram-positive normal flora of human skin, especially the skin of armpits, legs, between foot fingers, and face (1, 2). In recent decades, especially due to the increase in the employment of implants, this bacterium has been emerged as a predominant hospital pathogen and is considered as the third most common causes of hospital-acquired infections. The ability of this bacterium to form biofilms is its most significant characteristic in terms of pathogenesis (2, 3).

Biofilm generated by *S. epidermidis* can initially help escape host immune system through the inhibition of macrophage- and neutrophil-mediated phagocytosis. Also, biofilm can prevent the penetration of antibiotics to bacteria residing within the inner part of the structure. Therefore, in addition to acquiring antibiotic resistance genes (4-6), biofilm-producing bacteria can inhibit the direct penetration of antibiotics and therefore, inhibit their efficacy (6-8). Today, researchers believe that

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one of the most important causes of resistance and persistence of bacteria as well as strong ability to form biofilms is the presence of persister cells (9). Persister cells are a population of bacteria generated in response to high concentrations of antimicrobial compounds under harsh environmental conditions. These cells can tolerate these conditions without any genetic alterations with various mechanisms (9). These mechanisms include the employment of genes involved in SOS response, sigma factor S, alarm ones such as ppGpp, or toxin-antitoxin systems which are activated in stress conditions and inhibit vital bacterial activities such as translation and replication (10-13). Toxin-antitoxin (TA) systems consist of two genes on a single operon coding for a protein toxin and a protein or RNA antitoxin (14). It has been indicated that chromosomally-encoded TA systems are associated with cellular events such as programmed cell death, biofilm formation, persister cell formation in the presence of antibiotics, and genomic DNA stabilization (15). MazEF is one of the TA systems which is chromosomally encoded by bacteria and several studies have shown the important physiological role of MazF homologues (16). It has been indicated that persister cells are present within biofilm structures and are generally responsible for bacterial persistence and chronic infections despite antimicrobial treatments (13). Spore-forming probiotics are recently considered important both scientifically and commercially, among which Gram-positive *Bacillus* spp. are highly recognized and their employment has been increased due to their intrinsic ability to form endospores with high survival capabilities in harsh environmental conditions, metabolite formation, therapeutic potentials, and the ability to induce immune system antimicrobial properties (17, 18). Furthermore, their ability to secret antimicrobial compounds such as coagulin, amikacin, and subtilin can be used to combat pathogenic bacteria (18).

By utilizing spore-forming probiotics, it is hypothesized that they could effectively target and eliminate persister cells of *S. epidermidis*. The potential benefits of this approach include enhanced treatment efficacy by targeting persister cells, which are often responsible for recurrent infections, the employment of spore-forming probiotics could improve the effectiveness of antibiotic treatments and reduce the likelihood of treatment failure. Reduced antibiotic resistance: by specifically targeting persister cells, this approach may help minimize the development of antibiotic resistance in *S. epidermidis*, a common and problematic pathogen. Alternative treatment option: Spore-forming probiotics offer a potential alternative to traditional antibiotics, which could be particularly beneficial for individuals with antibiotic allergies or those who have developed antibiotic resistance (19-21). So, this study aimed to assess the presence and expression of genes involved in stress response in persister cells within the biofilm structure of *S. epidermidis* and the employment of spore-forming probiotics for their eradication.

Materials and Methods

Strains

In the current study, standard strains of *S. epidiermidis* ATCC 12228 and *B. coagulans* ATCC 31284 from the microbial bank of Iran University of Medical Sciences, Tehran, Iran were used.

Primer design, DNA extraction, and PCR

Genomic DNA extraction was carried out using DNA extraction kit (Roche, Germany). The presence of *rpoS* (Gene ID: 947210), *relA* (Gene ID: 50018568), and *mazF* (GeneID: 1058007) was investigated using specific primers designed with Oligo 7.60 and Primer 3 Web (Table 1). PCR reaction was carried out in a final volume of 25 µL (containing 12.5 µL Master Mix (Ampliqon Co., Denmark), 1 µL of each primer, and 1 µL bacterial DNA (template) and sterile distilled water) in 35 cycles including initial denaturation for 5 min at 95 °C, annealing for 1 min at 60 °C, elongation for 25 s at 72 °C, and final elongation for 5 in at 72 °C. PCR products were assessed using electrophoresis on 1% agarose gel.

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Table 1. Sequences of designed primers in this study.

Genes		Primer sequence (5′-3′)	Product size (bp)	Tm (°C)
RpoS	F	ACTGTCTTGCAGCTTCTTGT	100	59
	R	AGCCAAAAAGAGACTGGTGAA		
relA	F	CCTTTAGCACATCGTCTCGGA	131	60
	R	GCTTCGCGTTCACTACGTTT		
mazF	F	GCGGATTTATCACCAGTTCAAGG	149	60
	R	ACGTGGGTTGGTATTTTCGC		
16srRNA	F	CGAACACGTGCTTTGCTTGA	152	60
	R	CCCATACCTGGTCCAACTTCA		

Minimum inhibitory concentration (MIC) determination

Overnight culture of *S. epidermidis* with turbidity of 0.5 McFarland was prepared with a dilution of 1:20. Then, 10 µL of bacterial suspension was added to 96-well plate containing 100 µL Muller Hinton broth (Merck, Germany) with different concentrations (0.0625 µg/ml to 64 µg/ml) of gentamycin (Sigma Aldrich) and incubated for 18 h at 37 °C. After 18 h incubation, turbidity of wells was assessed for MIC determination (22, 23).

Persister cell assay

To induce persister cells, one colony of overnight *S. epidermidis* culture was added to 5 ml of LB (Luria-Bertani) broth (Merck, Germany) and incubated for 24 h. Then, 1:100 dilution of bacterial culture was prepared and incubated on a shaker until reaching the OD600 of 0.25. Bacterial culture was then exposed to 50X MIC of gentamycin and serial dilution was prepared 1, 3, 5, 7, and 24 h after the addition of antibiotic and inoculated on LB agar (Merck, Germany). After 24 incubation in 37 °C, colony numbers were counted to determine the number of persister cells.

Evaluation of biofilm formation using microtiter plate assay

First, samples were cultured on solid media and incubated for 24 h. Then, single colonies were inoculated to 5 ml of trypticase soy broth media (TSB) (Merck, Germany) containing 1% glucose and incubated for 24 h for 37 °C. Then, turbidity was modified according to 0.5 McFarland and 200 µL of the prepared suspension was added to the microplate wells and incubated for 18 h at 37 °C. Then, the supernatant of each well was discarded and each well was washed three times with sterile physiological serum. Next, plates were incubated for 1 h at 65 °C to remove the excess water and crystal violet staining was carried out for 15 min. The excess dye was discarded, and the plate was washed with water. Then, 100 µL of ethanol 95% was added to the wells and OD570 was recorded by ELISA reader (Oraganon Teknika, Netherlands). Biofilm formation was interpreted according to the OD of negative control (TSB containing 1% glucose) (24).

Anti-persister cell effects of spore-forming probiotics

B. coagulans ATCC 31284 was cultured anaerobically in Man-Rogosa-Sharpe (MRS) broth (Merck, Germany) and after centrifugation, supernatant was discarded and sterile filtered (0.2 µm filters). Dilutions of 1, 0.5, and 0.25 µg/ml were used to treat biofilm cells to induce persister cell formation and at different time intervals, serial dilution was prepared and inoculated on LB agar. Colonies were counted after 24 h incubation at 37 °C to determine the number of persister cells compared to the untreated cells.

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Statistical analysis

Data were analyzed using GraphPad Prims 8 software. T-test was used to assess the mean changes of gene expression compared to the control in presence of internal *16srRNA* gene as the internal control and $p<0.05$ was considered as significant.

Results

PCR results

To confirm the presence of the studied genes in *S. epidermidis*, PCR assay was carried out and according to the results the studied strain harbored all the studied genes (Fig. 1).

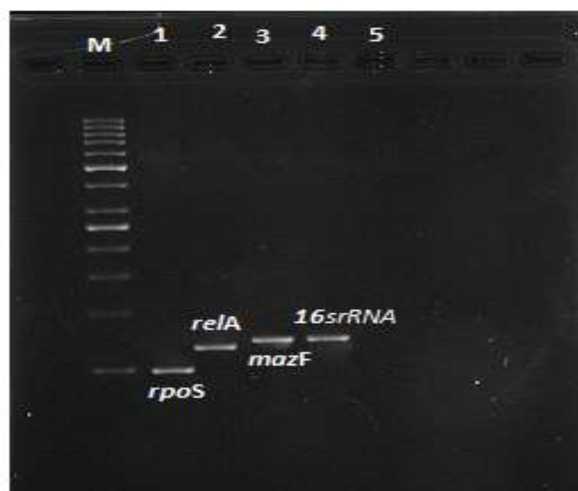

Fig. 1. Gel electrophoresis of the PCR products targeting the studied genes in *S. epidermidis*. M: Marker (100 bp), Lane 1: *rpoS* gene (100 bp), Lane 2: *relA* gene (131 bp), Lane 3: *mazF* gene (149 bp), Lane 4: *16S rRNA* gene (152 bp), NTC: Negative control.

Minimum inhibitory concentration (MIC)

MIC of gentamycin against *S. epidermidis* was 0.5 µg/ml based on the micro-dilution method.

Determination of persister cell count after exposure to Gentamycin

To induce persister cells, 100X MIC of gentamycin (50 µg/ml) was used. Colony count was done in triplicates and according to the results, after 3 h exposure of *S. epidermidis* to gentamycin, the number of viable cells decreased significantly ($p< 0.05$) and after 7 h, colony count remained the same and only persister cells survived ($p> 0.05$) (Fig. 2).

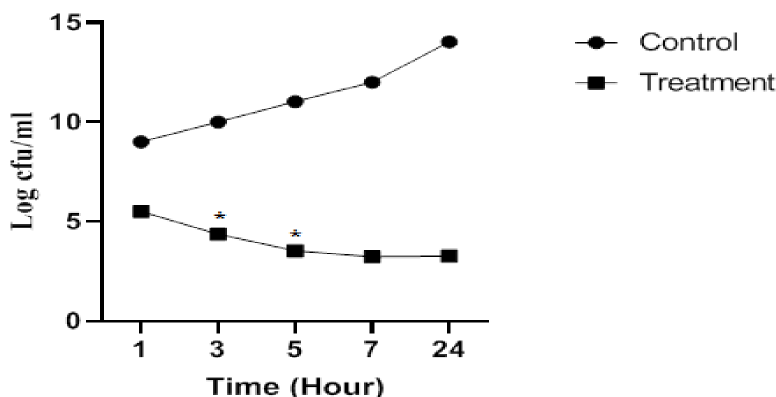

Fig. 2. The quantity of *S. epidermidis* persister cells post gentamycin exposure. Colony counts were performed in triplicate. Following 3-hour exposure to gentamycin, a notable decline in viable cells was observed. By the 7-hour mark, colony count plateaued, indicating survival of persister cells exclusively (*: $p < 0.05$).

Biofilm formation

The results of microtiter plate assay showed that the studied strain showed high capability of biofilm formation with an OD of 3.5.

Persister cells in biofilm structure

Colony count was carried out in triplicates and according to the results, after 5 h exposure of the biofilm to gentamycin, the

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number of bacterial colonies remained the same and only persister cells survived ($p > 0.05$). (Fig. 3).

Expression of the studied genes in biofilm and persister cells

The results of real-time PCR showed that the expression of *rpoS* gene increased 3.4, 4.7, and 46.7-fold at 2, 8, and 24 h after biofilm formation, indicating the potential role of this gene in the biofilm formation of *S. epidermidis*. Also, the expression of *relA* gene showed 3.9-fold increase in 2 h but at 8 and 24 h after biofilm formation, its expression was 1.7 and 1.2-fold, respectively, indicating that this gene might be involved in the establishment of biofilm structure. *mazF* expression was also increased compared to the control, however, its expression was higher at 8 h compared to 2 and 24 h (Fig. 4). Also, expression of *rpoS* and *relA* genes showed 5.6- and 6.8-fold increase in persister cells compared to the control ($p < 0.05$); however, *mazF* expression was not significantly different from the control ($p > 0.05$). Therefore, these results showed the possible role of *rpoS* and *relA* genes in the formation of persister cells in *S. epidermidis*.

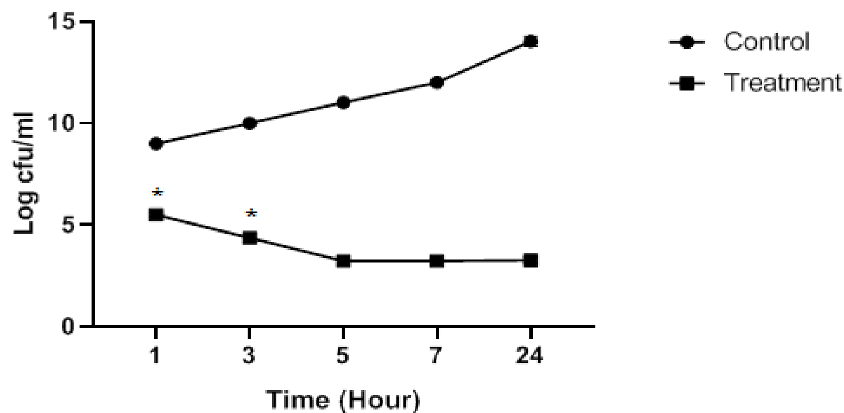

Fig. 3. Quantification of persister cells within the *S. epidermidis* biofilm following gentamycin exposure. Following a 5-hour exposure period, the bacterial colony count within the biofilm remained constant, with only persister cells exhibiting survival (*: $p < 0.05$).

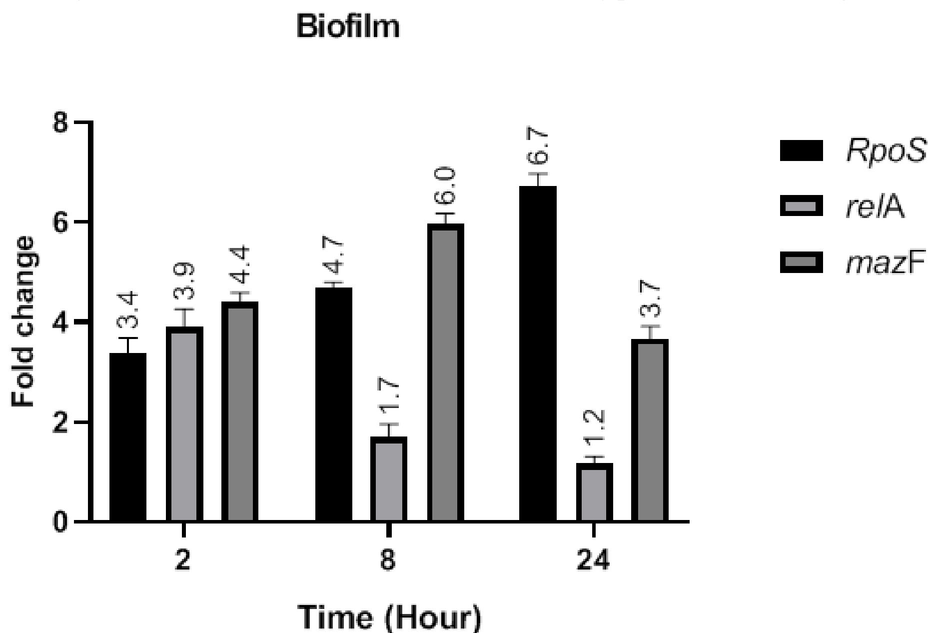

Fig. 4. Gene expression profiling within the *S. epidermidis* biofilm compared to control conditions. Real-time PCR analysis revealed significant upregulation of the rpoS gene, with fold changes of 3.4, 4.7, and 46.7 at 2-, 8-, and 24-hours post-biofilm formation, suggesting its pivotal role in biofilm development. Additionally, the relA gene exhibited a 3.9-fold increase at 2 hours, followed by 1.7-fold and 1.2-fold changes at 8 and 24 hours, respectively, implicating its potential involvement in early biofilm establishment. Notably, mazF expression was elevated across all time points, with peak expression observed at 8 hours compared to 2 and 24 hours.

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Effect of spore-forming probiotic on persister cells

According to the results of persister cells colony count, 1 µg/ml of the supernatant could decrease persister cell numbers for log2.4 as compared to the control (untreated cells) ($p < 0.05$). Also, at the concentration of 0.5 µg/ml, decreased number of persister cells for log0.8 was observed ($p < 0.05$), however, this decrease was not observed at concentration of 0.25 µg/ml ($p > 0.05$).

Discussion

In the present study, persister cell formation and the expression of *rpoS*, *relA*, and *mazF* genes were evaluated and, in the end, the effect of the probiotic *B. coagulans* probiotic was assessed on persister cell formation of *S. epidermidis.* The results showed that high concentration of gentamycin (50 µg/ml) leads to persister cell formation after 7 h and these cells emerged in bacterial biofilm in the aforementioned condition after 5 h. These results indicated that antimicrobial compounds could induce persister cells at high concentrations and therefore, one of the reasons for the persistence of this bacterium on hospital surfaces and other health care centers despite proper measures is the presence of persister cells, especially in the biofilm structure of this bacterium.

According to the results of real-time PCR, expression levels of *rpoS* and *mazF* genes were significantly higher compared to *relA* during biofilm formation when compared to the control. Therefore, these genes might be involved in biofilm formation of this bacterium. In a study by Shivaee et al. on the expression of different genes in *S. epidermidis*, *mazF* was expressed at different time points during biofilm formation; however, its expression was higher at the beginning of biofilm formation. They also reported that *altE* and *sdrH* genes were involved in the formation and development of biofilm structure (24) which was in accordance with the current study. In 2017, Kato et al. showed that the deletion of *mazF* gene in *S. aureus* leads to increased biofilm formation through *ica*-dependent pathway, and that this pathway could be involved in biofilm formation through the interference with *mazF* activity (25). These results and the result of the current study show that pathways involved in biofilm formation and *mazF* activity might be different in *S. aureus* and *S. epidermidis*. According to the literature, MazEF affects biofilm formation and its persistence through the induction of programmed cell death (26). The expression of *rpoS* also showed a significant increase in the biofilm structure. In general, *rpoS* gene, which is considered as a vital regulator in the stationary phase and in response to stress responses in Gram-negative bacteria, have not been investigated intensively in *S. epidermidis*. In the current study, this gene was investigated in biofilm formation and the results showed its elevated expression levels at 2, 8, and 24 h after biofilm formation compared to the control for 3.4, 4.7, and 6.7-fold, respectively, indicating the potential role of this gene in the development of *S. epidermidis* biofilm. Moreover, *relA* gene showed its highest expression level at 2h after biofilm formation, while its expression was reduced after 8 and 24 h, indicating its potential role in the establishment of the biofilm structure. However, more studies are required to investigate the role of these genes in biofilm formation.

Increased expression of *relA* and *rpoS* genes was observed in persister cells compared to the control. However, the expression of *mazF* gene did not change significantly compared to the control. According to these results, *rpoS* and *relA* genes may contribute to persister cell formation by *S. epidermidis*. One of the known mechanisms leading to the formation of persister cells is the activation of *relA* gene. In fact, this gene possibly inhibits protein formation in bacteria and leads to persister cell formation through the activation of ppGpp, which is considered as a signal in conditions of amino acid depletion. The results of this study also confirmed this hypothesis. It has been suggested that MqsA antitoxin facilitates bacterial survival under oxidative stress by influencing RpoS, which is an

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important factor in bacterial oxidative stress response (27).

The effect of *B. coagulans* supernatant was also investigated on persister cell formation and according to the results, the supernatant of this probiotic which contains bacterial metabolites can significantly reduce the number of persister cells in high concentration. Many studies have reported the effect of probiotics and their metabolites on pathogenic bacteria (28-30). However, in general, we could not find a study on the effect of probiotics and their metabolites on persister cell formation to compare our results. Owing to the potential sensitivity of persister cells to probiotics, probiotic-based therapeutic approaches can be taken into consideration in the future.

The results of the current study showed the induction of *S. epidermidis* persister cells in biofilm structures after exposure to high doses of antibiotic. Therefore, the employment of high concentrations of antimicrobial compounds can lead to the persistence of these bacterial populations and hence recurrence of infection. Various genes are involved in persister cell formation and further investigations, including deletion of these genes, can help us understand their precise role in this process. Furthermore, the results of current study showed that the supernatants of probiotic bacteria contain their secretive metabolites, and the isolation of these metabolites can be helpful in future clinical and research studies.

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Conflict of Interest

None to be declared.

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