



# Cell-Free Culture Supernatants of Lactobacilli Modify the Expression of Virulence Factors Genes in *Staphylococcus aureus*

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Received 2019 July 31; Revised 2019 December 20; Accepted 2020 January 23.

## Abstract

**Background:** Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant microorganism and the predominant nosocomial pathogen all over the world. The potential benefits of probiotic lactobacilli against pathogenic bacteria have been shown in many studies.

**Objectives:** This study aimed to investigate the effect of cell-free culture supernatant (CFS) of probiotic *Lactobacillus* spp, including *Lactobacillus reuteri*, *L. plantarum*, and *L. fermentum*, on virulence factor gene expression of MRSA.

**Methods:** Lactobacilli were cultured in MRS and the cells were harvested by centrifuging at  $10,000 \times g$  for 10 min at 4°C. The pellet was discarded and 1/2 and 1/4 CFS concentrations from *Lactobacillus* spp were added to the medium (MHB) containing  $10^7$  CFU/mL of the MRSA strain. A quantitative polymerase chain reaction (qPCR) was performed to measure the expression of virulence factors at the transcriptional level.

**Results:** The results showed that lactobacilli CFS had no obvious inhibitory effects on the growth of *S. aureus*. The qPCR assay showed that the expression levels of *sea*, *sae*, *agr A*, *tst*, *spa*, and *spi* genes reduced at different levels, depending on the concentration of CFS and the species of lactobacilli so that the maximum significant down-regulation rate was observed in the *sea* and *tst* genes (up to 23.5 folds in the presence of 1/2 concentration of *L. reuteri* CFS after 12 h incubation).

**Conclusions:** Cell-free culture supernatants of probiotic bacteria can down-regulate the virulence genes. Consequently, toxins and enzymes are less produced by *S. aureus* as a food-borne pathogen. Therefore, the presence of CFS in the food probably reduces diarrhea and vomiting caused by *S. aureus*.

**Keywords:** *Lactobacillus*, MRSA, Virulence Factors, Gene Expression

## 1. Background

*Staphylococcus aureus* is one of the most frequent foodborne pathogens causing various infections such as wound infection, septicemia, meningitis, toxic shock syndrome (TSS), and osteomyelitis in humans (1, 2). *Staphylococcus aureus* produces different toxins and enzymes including enterotoxins, DNase, coagulase, lipase, TSST-1 (*tst*), and hemolysin involved in the pathogenesis of the diseases (3). The enterotoxins (*sea* to *see* and *seg* to *sej*) are heat-resistant (4, 5). These toxins can cause food poisoning, staphylococcal gastroenteritis, and diverse autoimmune and allergic diseases (6, 7). *Staphylococcus aureus* also secretes protein A (*spa*) and second immunoglobulin-binding protein (*sbi*), virulence factors associated with staphylococcal pneumonia, and hypodermic infection (8, 9). Therefore, the *sea*, *spa*, *tst*, and *sbi* genes encode impor-

tant virulence factors that are regulated by accessory gene regulators (*agr*) and two-component signal transduction system (*sae*) in response to environmental stimulating factors (10-12).

Methicillin-resistant *S. aureus* (MRSA) like other multidrug-resistant bacteria is now resistant to many antimicrobial agents and cause serious infections in inpatients and outpatients (6, 13). These isolates from human sources and dairy products are related to foodborne diseases (e.g., diarrhea) in humans who consume contaminated dairy products. Therefore, there is an increasing need to study alternative agents with maximum efficacy and minimum side effects (14, 15).

Probiotic bacteria like *Lactobacillus* species (*Lactobacillus reuteri*, *L. fermentum*, and *L. plantarum*), as a beneficial group of microbiota, play a considerable role in hu-

man health (16, 17). Lactic acid bacteria (LAB) demonstrate the inhibitory and therapeutic effects on some intestinal pathogenic infections, antibiotic-associated gastrointestinal complications, diarrhea, allergies, cancers, and certain inflammatory and immunomodulatory disorders (18-20). The supernatant of the LAB culture includes aroma components, fatty acids, organic acids, low-molecular-mass compounds, and hydrogen peroxide that can kill pathogens (21). In recent studies, the effect of the supernatant of LAB has been probed on the inhibition of the growth of pathogenic bacteria such as *S. aureus* and less attention has been put on the effect of the supernatant on the expression of virulence factors.

## 2. Objectives

In this study, we evaluated the effect of the CFS of three probiotic LAB (*L. reuteri*, *L. fermentum*, and *L. plantarum*) on the gene expression regulator system (*sae* and *agr A*) and other genes (*sea*, *tst*, *spa*, and *sbi*) at the transcriptional level.

## 3. Methods

### 3.1. Bacterial Strains and Growth Conditions

The Methicillin-resistant *S. aureus* (MRSA) strain (ATCC 33591), *L. reuteri* (ATCC 23272), *L. plantarum* (ATCC 8014), and *L. fermentum* (ATCC 9338) were obtained from the Persian type culture collection (PTCC). Methicillin-resistant *S. aureus* was cultured in Muller Hinton Broth (MHB) at 37°C until it reached the exponential phase of growth. Lactobacilli were cultured in MRS (De Man, Rogosa, and Sharpe) broth at 37°C until they reached the stationary phase of growth. After a 24-h period, the lactobacilli cells were harvested by centrifuging at  $10,000 \times g$  for 10 min at 4°C. The pellet was discarded and the cell-free supernatants (CFS) were then adjusted to natural pH (pH = 7) with NaOH (1 N) and sterilized through a 0.22- $\mu\text{m}$  filter. These supernatants were stored at -20°C for later use.

### 3.2. Microbial Interactions

The density and the number (CFU/mL) of MRSA in cultures were measured through the use of spectrophotometry at OD600. The 1/2 and 1/4  $\times$  CFS of Lactobacilli spp. were added to the medium (MHB) containing  $10^7$  CFU/mL of MRSA bacteria. After 6 and 12 h of incubation, the bacterial density was quantified at OD600, and then centrifuged at  $12,000 \times \text{rpm}/10$  min at 4°C. The supernatants were removed and the pellet was used for RNA extraction. A lactobacilli-free culture of MRSA was used as control at the logarithmic phase of growth.

### 3.3. RNA Extraction and cDNA Synthesis

The RNA was extracted using a Rapid Bacterial RNA Isolation Kit (Bio Basic, Inc., Canada) according to the manufacturer's instructions. The total RNA was solved in 30  $\mu\text{l}$  of RNase-free water. To remove all DNA, the purified RNA was treated with DNase I, RNase-free (Cinna Gene, Iran) after extraction. The purity of RNA was determined by calculating A260/A230 and A260/A280 ratios (Denovix, NanoDrop, USA) and the integrity of total RNA was assessed with 1% agarose gel (data not shown). The cDNA was synthesized using a cDNA reverse transcription kit (Pars Tous, Iran).

### 3.4. Quantitative PCR

The quantitative polymerase chain reaction (qPCR) was performed using primers described previously (22-24). Briefly, all PCR assays were accomplished in a total volume of 20  $\mu\text{L}$ , containing 10  $\mu\text{L}$  of Sybr Green Mix (Takara Bio®, Japan), 0.1  $\mu\text{M}$  of primers, 5 ng of cDNA, and deionized water. The RT-qPCR amplification was carried out by the Applied Biosystems 7500 and the Step One Plus Real-time PCR system (ABI, France). The cycling parameters for qPCR included an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. We used LinRegPCR software to calculate the amplification efficiency of each sample (25). We entered the raw dataset of RT-qPCR outputs into LinRegPCR version 2017.1 software (<https://LinRegPCR.HFRC.nl>). The performance and efficacy of all primers was evaluated separately on the qPCR data of samples. The housekeeping gene 16S rRNA was applied as an internal control. The expression ratio (fold) and the up-regulation and down-regulation of genes were calculated using the  $\Delta\Delta\text{Ct}$  formula. The data were represented as mean  $\pm$  standard deviation (SD). All experiments were accomplished independently at least three times. Statistical analysis was performed using GraphPad Prism version 5.00 software (GraphPad software, Inc., CA, USA).

## 4. Results

While the growth of MRSA in the presence of CFS of lactobacilli was not inhibited over the six and 12-h periods of post-treatment, the level of gene expression was significantly reduced (Table 1) depending on the species of *Lactobacillus*, the supernatant concentration, and the incubation time.

### 4.1. Gene Expression at 6-h Post-Treatment

The attenuation of virulence gene expression of MRSA was observed at 6-h post-treatment (Figure 1). At this primary time-point, no significant change was observed in

**Table 1.** Growth of *Staphylococcus aureus* (MRSA) for up to 12 h in the Presence of the CFS of Various Species of Lactobacilli<sup>a</sup>

Species	Bacterial Growth at OD600		
	0 h <sup>b</sup>	6 h	12 h
<i>Staphylococcus aureus</i> (MRSA)	0.41	0.71 ± 0.021	0.986 ± 0.011
+ <i>Lactobacillus reuteri</i> (CFS)	0.41	0.67 ± 0.031	0.88 ± 0.042
+ <i>L. plantarum</i> (CFS)	0.41	0.656 ± 0.007	0.845 ± 0.012
+ <i>L. fermentum</i> (CFS)	0.41	0.63 ± 0.01	0.82 ± 0.026

<sup>a</sup>Values are expressed as mean ± SD.<sup>b</sup>Starting bacterial inoculum.

the expression of any of the study genes (Figure 1A, E, F, B, and C). By increasing the CFS concentration to  $1/2 \times$  CFS, the ratio of gene expression decreased significantly from 2.18 to 7.98 folds compared to that of the control in the *spa*, *sea*, *sae*, *tst*, and *agr* A genes in the presence of the CFS of all the three Lactobacilli studied ( $P < 0.05$ ). The maximum reduction belonged to the *sea* gene (7.89 folds) (Figure 1C). However, by increasing the concentration of *L. reuteri* and *L. fermentum* CFS, there was no significant decrease in the expression of the *sbi* and *spa* genes (Figure 1E and D).

#### 4.2. Gene Expression at 12-h Post-Treatment

The expression levels of the studied genes also decreased at 12-h post-treatment. However, they were different from the expression levels at 6-h post-treatment (Figure 2). The expression ratios of the *agr* A, *tst* and *sea* genes in all types of CFS markedly decreased 12-h post-treatment in comparison with the control group at a concentration of  $1/2 \times$  CFS from 14.3 to 26.6 folds (Figure 2A-C). The significant reductions in the expression of the *sae* gene were also observed in the presence of *L. plantarum* and *L. reuteri* CFS (Figure 2F). However, by increasing the incubation time, *L. fermentum* CFS ( $1/2$  and  $1/4$ ) could not significantly affect the expression ratios of the *sae*, *sbi*, and *spa* genes (Figure 2F and E). The maximum reduction, up to 23.5 folds, was observed in the *tst* and *sea* genes (Figure 2B and C).

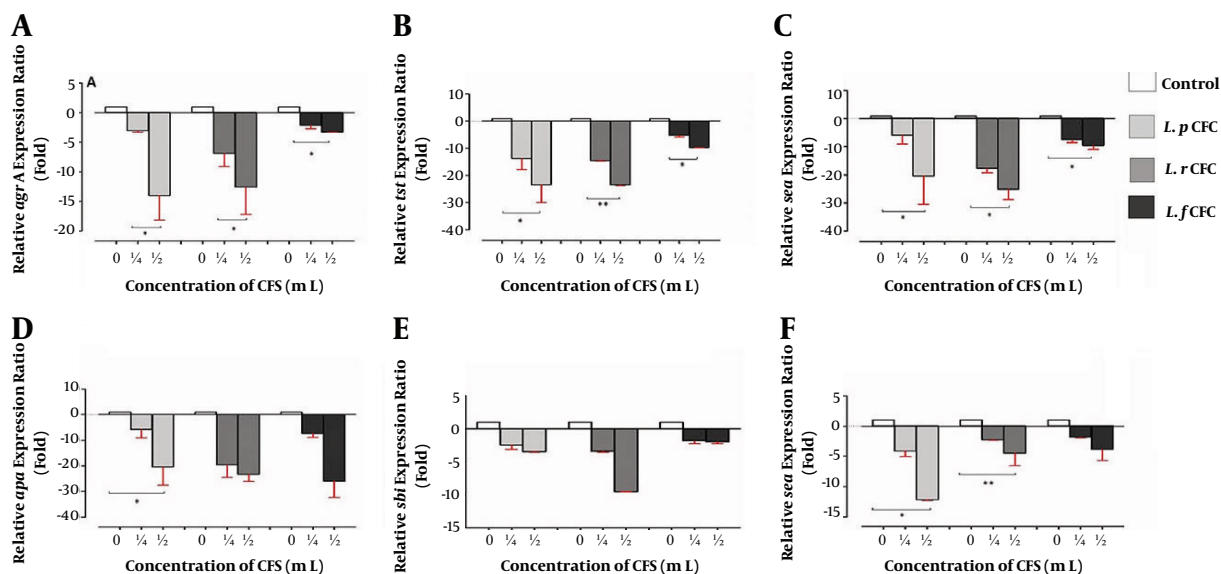
## 5. Discussion

Despite various studies conducted on the virulence genes of *S. aureus* aiming to reduce its adverse effects, this human pathogen is still considered an extremely prevalent pathogen worldwide (26, 27). The objective of this study was to compare the potential effects of CFS from three different probiotic lactobacilli including *L. reuteri*, *L. fermentum*, and *L. plantarum* on the expression of some virulence factor encoding genes of MRSA at different incubation times and concentrations. Lactobacilli spp. produce a

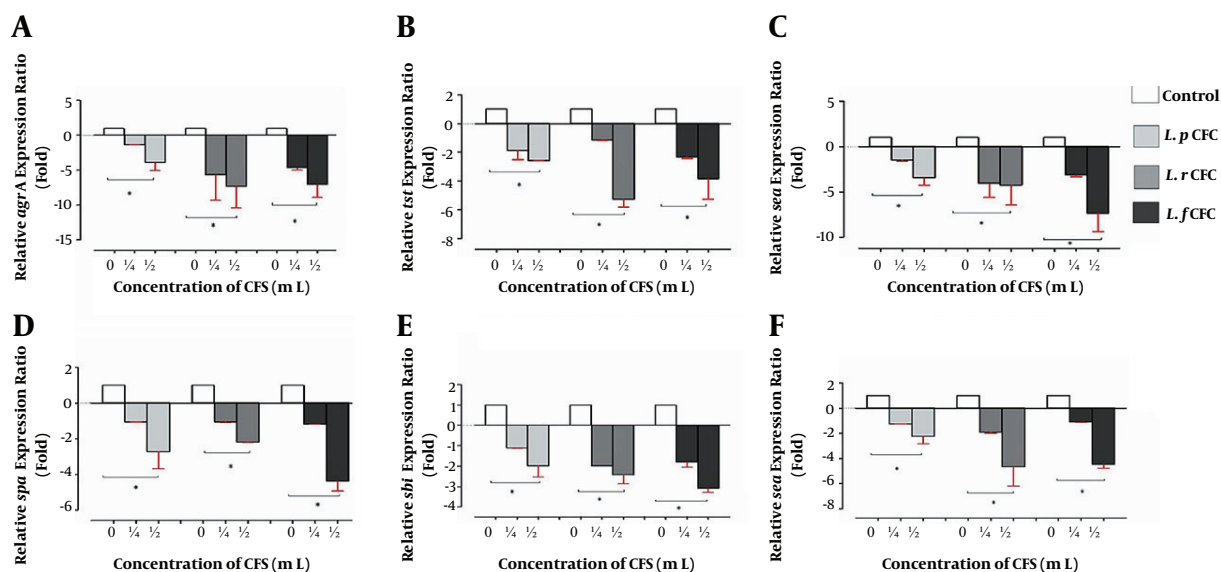
class of molecules with antimicrobial capacity (28). For example, bacteriocins produced by *L. fermentum* can reduce adhesion and cytotoxicity properties of MRSA (29). The CFS of *L. salivarius* and *L. fermentum* can inhibit the biofilm formation of *S. aureus* (30). The complete inhibition of *Salmonella* growth has been shown in the presence of the CFS from *L. johnsonii*, *L. rhamnosus*, *L. casei*, and *L. plantarum* (31, 32). *Lactobacillus reuteri*, *L. plantarum*, and *L. fermentum* have been extensively investigated for their probiotic effects on pathogenic bacteria (27, 33-35).

Vahedi-Shahandashti et al. (36) stated that some probiotic lactobacilli culture supernatants had an inhibitory effect on *Serratia marcescens* swarming and antibiotic resistance. Hiawy et al. in Iraq showed that the CFS of *L. fermentum* has inhibitory effects on the growth rate and expression reduction of the gene responsible for biofilm formation in MRSA (21). Our results indicated that *L. reuteri*, *L. fermentum*, and *L. plantarum* reduced the expression of the *tst*, *sae*, and *sea* genes, as well as the *agr* quorum-sensing system, in MRSA without any tangible effects on its growth. The *agr* A system is an important global regulatory system that controls the expression of many genes associated with secreting proteins and virulence determinants including protease and collagenase in *S. aureus* (37, 38). Based on our results and in parallel with the Li et al.'s (39) investigations, it is proven that the reduction in the expression of virulence factors is related to the reduction in the *agr* system. In fact, the *agr* A gene is down-regulated at the transcriptional level by CFS. Consequently, it reduces the expression of the *sae*, *tst*, *sea*, *sbi*, and *spa* genes. In parallel with other similar studies, it seems that the *tst* gene is regulated by the *sae* and *agr* systems (40).

The results showed that the significant down-regulation of gene expressions was in the preference of the  $1/2 \times$  CFS concentration while none of the genes was significantly altered at the low concentration of CFS ( $1/4 \times$  CFS). It has been proven that the sub-lethal dosage of these antimicrobial compounds can reduce the expression of virulence-associated genes in *S. aureus* and *Vibrio cholera*, without affecting their growth (7, 39, 41-43). These results reveal that increasing the incubation time causes a significant reduction in the gene expression so that a significant reduction in the *sbi* and *spa* gene expressions achieved by increasing the incubation time. In addition, the CFS of these species of LAB was effective on the level of gene expression. Our findings showed that the CFS of *L. reuteri* and *L. plantarum* had a more powerful effect than the CFS of *L. fermentum* on gene expression. Previous researches suggested that human probiotic isolates, such as *L. plantarum*, inhibited bacterial virulence, inhibited bacterial virulence, representing a promising alternative to antibiotic prophylaxis of staphylococcal menstrual



**Figure 1.** Relative gene expression ratios of MSRA in the presence of CFS of *Lactobacillus* spp. after 6-h incubation. A, *agrA* gene; B, *tst* gene; C, *sea* gene; D, *spa* gene; E, *sbi* gene; and F, *sae* gene, at two different concentrations of three different CFS. Data are the average of three independent experiments performed in triplicate and reported as fold  $\pm$  SD.



**Figure 2.** Gene expression of MSRA in the presence of CFS of *Lactobacillus* spp. after 12-h incubation. A, *agrA* relative expression ratio; B, *tst* relative expression ratio; C, *sea* relative expression ratio; D, *spa* relative expression ratio; E, *sbi* relative expression ratio; and F, *sae* relative expression ratio at two different concentrations of three different CFS after 12 hours. Data are the average of three independent experiments performed in triplicate and reported as fold  $\pm$  SD.

TSS and potentially of other *S. aureus*-mediated diseases (40, 44). Though the actual physiological function of supernatant compounds is unclear, these compounds may serve signaling molecules involved in host-bacterial interactions, considering their biological effects in humans

(39, 45).

It is necessary to mention that *L. fermentum* is not recognized as a well-known probiotic bacterium. It had an inhibitory effect on virulence gene expression, depending on concentration (1/2 more effective than 1/4 CFS concen-



tration) and incubation time. It seems that a reduction in gene expression is associated with the pathogenesis and consequently their supernatant component is due to the down-regulation of virulence genes. Therefore, the compounds found in CFS can influence the *agr*, *sae*, *sea*, *tst*, *sbi*, and *spa* genes to reduce expression at the transcription level.

### 5.1. Conclusions

Probiotic bacteria and their products in food can inhibit the growth and down-regulate virulence gene expression. Consequently, toxins (enterotoxins) and enzymes are less produced by *S. aureus* as a bacterium causing vomiting. Therefore, the presence of CFS in the food probably reduces diarrhea and vomiting caused by bacteria.

### Acknowledgments

This project was funded by a grant (no.: 9.3692) from the Vice Chancellery for Research and Technology, Rafsanjan University of Medical Sciences (RUMS). The authors thank the RUMS Molecular Medicine Research Center for kindly providing the required equipment.

### Footnotes

**Authors' Contribution:** Study concept and design: Mohammad Zare-Bidaki and Vahid Bagheri. Analysis and interpretation of data: Mahnaz Ramezani. Drafting of the manuscript: Mahnaz Ramezani. Critical revision of the manuscript for important intellectual content: Nahid Zainodini, Hamid Hakimi, Ebrahim Rezazadehazarandi, and Reza Bahramabadi. Statistical analysis: Mahnaz Ramezani.

**Conflict of Interests:** There is no conflict of interest declared.

**Ethical Approval:** The ethical approval was 9.3692.

**Funding/Support:** Rafsanjan University of Medical Sciences (RUMS) funded the study.

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