The Inhibitory Effects of 2 Commercial Probiotic Strains on the Growth of *Staphylococcus aureus* and Gene Expression of Enterotoxin A

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**Abstract**

**Background:** Food-borne intoxications are current problems in human society and most of them are caused by the enterotoxins of *Staphylococcus aureus*. Staphylococcal enterotoxin A (SEA) is the most frequently responsible for staphylococcal food poisoning outbreaks. From a food safety and human health point of view, lactic acid bacteria (LAB) may provide a promising strategy to combat the pathogenic bacteria, particularly *S. aureus*.

**Objective:** The objective of this study was to evaluate the inhibitory activity of two commercial lactobacillus strains on growth and enterotoxin A production by *S. aureus*. Moreover, the inhibitory effect of these strains on gene expression of enterotoxin type A was assessed using real-time Polymerase chain reaction (PCR).

**Materials and Methods:** In this study the inhibitory effect of two commercial probiotic strains, *Lactobacillus acidophilus* (LA5) and *Lactobacillus casei* 01 on the growth and enterotoxin production of *S. aureus* was evaluated at 25 and 35°C. The gene expression of SEA of *S. aureus* was also evaluated by real time (RT) PCR technique.

**Results:** The lactobacillus strains decreased the bacterial count at both temperatures compared with the control group. This reduced effect was greater at 25°C (3 log/CFU) than 35°C (2 log/CFU). The production of SEA, SEC and SEE was inhibited by the lactobacillus strains. Furthermore, the gene expression of SEA was significantly suppressed in *S. aureus* co cultured with studied lactobacillus strains and the greatest down-regulation of sea (10.31 fold) was observed in co-incubation of *S. aureus* with LC01 at 25°C.

**Conclusion:** This research raises important implications for the potential use of LAB as a natural preservative in foodstuffs by correct microbial ecology of the environment and a new approach for biocontrol of *S. aureus*.

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**Background**

Food-borne intoxications are current problems in human society and most of them are caused by the enterotoxins of *Staphylococcus aureus*.1 Staphylococcal food poisoning (SFP) is due to the production of staphylococcal enterotoxins (SEs) by *Staphylococcus aureus* strains contaminating foodstuffs such as meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, particularly cream-filled pastries and cakes, and sandwich fillings.2,3 The control of this disease is of social and economic importance as it represents a considerable burden in terms of loss of working days and productivity, hospital expenses, and economic losses in food industries, catering companies and restaurants.2,4

SEs are synthesized throughout the logarithmic phase of growth during the transition from exponential to stationary phase. They are active in high nanogram to low microgram quantities and are resistant to physical conditions (heat treatment and low pH) that easily destroy the bacteria that produce them as well as proteolytic enzymes, and hence retain their activity in the digestive tract after ingestion.2,5 Staphylococcal enterotoxin A (SEA) is the most frequently responsible for staphylococcal food poisoning outbreaks. The expression of virulence factors in *S. aureus* is tightly controlled by a complex network of regulatory systems. Genes encoding SEs are carried on various genetic supports, most of which are mobile genetic elements including phages (*sea*, *sec* and *sep*) and plasmids (*sed*, *sej*, *ser*, *ses* and *set*).6 However, the *sea* gene, carried in the bacterial genome by a polymorphic family of temperate bacteriophages, which is composed of 771 base pairs and encodes enterotoxin A precursor of 257...
The inhibition of *S. aureus* growth and production of SE in foodstuffs is of importance in the public health, therefore, to detect the prevalence of enterotoxic strains in foods is required. From a food safety and human health point of view, lactic acid bacteria (LAB) may provide a promising strategy to combat against *S. aureus*. In recent years interactions between *S. aureus* and LAB have been examined in several ecosystems, including fermented foodstuffs as well as the nasal and vaginal environments. However, studies of the inhibition of *S. aureus* virulence expression by LAB, including the inhibition of SE production, are quite scarce. Few studies have described the inhibition of enterotoxin production in the presence of LAB, and none have unraveled the mechanisms involved in such antagonism.

### Objectives

The objective of this study was to evaluate the inhibitory activity of 2 commercial *Lactobacillus* strains - *Lactobacillus acidophilus* (LA5) and *Lactobacillus casei* 01 - on growth and enterotoxin A production by *S. aureus* in mixed cultures. Moreover, the inhibitory effect of these strains on gene expression of enterotoxin type A was assessed using real time polymerase chain reaction (PCR).

### Materials and Methods

#### Bacterial Strains

*Staphylococcus aureus* ATCC 29213 (enterotoxin A producer) obtained from the culture collection of the Pasteur Institute, Tehran, Iran was used in this study. Two probiotic strains, *L. acidophilus* (LA5) and *L. casei* 01, were obtained from the Christian Hansen company.

#### Preparation of Inocula

*Staphylococcus aureus* was sub-cultured in Trypticase Soy Broth (TSB) and incubated for 18 hours at 35°C. The bacterial suspension was adjusted to an optical density (OD) of 0.1 at 600 nm using a Spectronic 20 spectrophotometer (Milton Roy Company, Houston, USA). This adjustment gave a cell concentration of 10⁷ CFU/mL as determined from previously prepared standard curve data. The number of cells in the suspension was enumerated by duplicate plating from 10-fold serial dilutions on BHI agar and counting the colonies after 24 hours incubation at 35°C. *Lactobacillus* cultures were prepared by sub-culturing in MRS (de Man, Rogosa and Sharpe) broth incubated for 24 hours at 35°C and similarly titrated on MRS agar.

#### Co-culture of *Staphylococcus aureus* and *Lactobacillus* Commercial Strains

Preparation of co-cultures of *Lactobacillus* strains and *S. aureus* was done as described by Laughton et al with some modifications. Each *Lactobacillus* strain (10⁷ CFU/mL) was grown in 10 mL TSB followed by inoculating *S. aureus* (10⁵ CFU/mL) into the medium. TSB medium inoculated with *S. aureus* or *Lactobacillus* individually were considered to be controls. Cultures were incubated at 25 and 35°C for 0, 24, 48 and 72 hours. Bacterial titres were determined by surface plate counting of aliquots of tenfold serial dilutions spread on MRS agar and Baird Parker agar plates for *Lactobacillus* strains and *S. aureus*, respectively.

#### Detection of Staphylococcal Enterotoxins

The RIDASCREEN SET kit (R-Biopharm GmbH, Darmstadt, Germany), a commercial SE visual immunoassay kit, was used to evaluate the presence of SEs. The kit utilizes monovalent capture antibodies against SEs types A to E. Detection and identification of the enterotoxin types was performed as recommended by the manufacturer.

#### RNA Extraction and cDNA Synthesis

*Lactobacillus* strains were co-cultured in TSB with *S. aureus* ATCC 29213 at both 35°C and 25°C for 24 hours. The effect of *Lactobacillus* commercial strains on gene expression of SEA was evaluated by real-time PCR (RT-PCR). Briefly, bacterial cultures were centrifuged in polypropylene tubes at 12,000 x g for 5 minutes at 4°C. The supernatant was removed and total RNA from bacterial cells was extracted using TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. RNA quality was monitored by measuring the absorbance at 260 and 280 nm (A260/280 ratio) using a NanoDrop spectrophotometer (Thermo scientific Nanodrop, Wilmington, USA). Synthesis of cDNA from 1-1.5 ng of RNA was conducted using the RevertAid First Strand cDNA synthesis kit (Fermentase, St. Leon-Roth, Germany) with random hexamer primers according to the manufacturer's instructions. For each RNA sample, a non-reverse transcribed control (NRTC) was included to detect contaminating genomic DNA. The cDNA synthesis was performed in a DNA Engine ABI thermocycler 2720 (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 65°C for 5 minutes, 42°C for 60 minutes and followed by 70°C for 5 minutes. Briefly, 1-1.5 ng of RNA was reverse transcribed with 1 mL random hexamer primers, 10 µM of each dNTP (2 µL), 4 µL 5x first strand buffer, 1 µL Ribolock (RNase inhibitor 20 U/µL), and 1 µL RevertAid™ M-MuLV reverse transcriptase (200 U/µL). For each RNA sample a NRTC was included.

#### Real-Time Polymerase Chain Reaction

RT-PCR was used to assess SEA gene expression using 16s rRNA as the endogenous control. The cDNA (1 µL) was used as a template for amplification in 20 µL final volume, containing 10 µL of power SYBR Green® II PCR master mix (Primer design, Southampton, UK), 0.5 µL of each primer design, 0.5 µL of each primer, and 10 µL of Power SYBR® Green PCR Master Mix (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The reaction mixture was subjected to 40 cycles of amplification; 95°C for 15 seconds and 60°C for 60 seconds.

### Results

Expression of SEA was evaluated by real-time PCR (RT-PCR). The effect of *Lactobacillus* commercial strains on gene expression of SEA was evaluated by real-time PCR (RT-PCR). Briefly, bacterial cultures were centrifuged in polypropylene tubes at 12,000 x g for 5 minutes at 4°C. The supernatant was removed and total RNA from bacterial cells was extracted using TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. RNA quality was monitored by measuring the absorbance at 260 and 280 nm (A260/280 ratio) using a NanoDrop spectrophotometer (Thermo scientific Nanodrop, Wilmington, USA). Synthesis of cDNA from 1-1.5 ng of RNA was conducted using the RevertAid First Strand cDNA synthesis kit (Fermentase, St. Leon-Roth, Germany) with random hexamer primers according to the manufacturer's instructions. For each RNA sample, a non-reverse transcribed control (NRTC) was included to detect contaminating genomic DNA. The cDNA synthesis was performed in a DNA Engine ABI thermocycler 2720 (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 65°C for 5 minutes, 42°C for 60 minutes and followed by 70°C for 5 minutes. Briefly, 1-1.5 ng of RNA was reverse transcribed with 1 mL random hexamer primers, 10 µM of each dNTP (2 µL), 4 µL 5x first strand buffer, 1 µL Ribolock (RNase inhibitor 20 U/µL), and 1 µL RevertAid™ M-MuLV reverse transcriptase (200 U/µL). For each RNA sample a NRTC was included.

RT-PCR was used to assess SEA gene expression using 16s rRNA as the endogenous control. The cDNA (1 µL) was used as a template for amplification in 20 µL final volume, containing 10 µL of power SYBR Green® II PCR master mix (Primer design, Southampton, UK), 0.5 µL of each
primer and 8 µL nuclease-free water. Forward and reverse sea primers were 5'-TTGGAAACGGTTAAACGAA-3’ and 5’-GAACCTTCCCATGAAAAACA-3’, respectively. Forward and reverse primers for the reference 16s rRNA gene were 5’-CAGCCCTGGGGAGTACG-3’ and 5’-AAGGTTGCGTCTTGTC3’, respectively.11 RT-PCR conditions were as follows: initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by melting curve analysis at 65-95°C (temperature transition rate of 0.1°C/s) based on continuous fluorescence reading. Fluorescence data were collected at the end of each cycle on a quantitative PCR system (Rotorgene -6000 Corbett, Sydney, Australia). The relative expression of the sea gene was calculated versus the calibration sample and the endogenous control (16s rRNA) to normalize the sample input amount, and the levels of sea expression of treated and untreated samples were compared. All determinations were done in triplicate.

Statistical Analysis
All experiments were repeated three times. Bacterial growth curves were drawn using GraphPad Prism 4 software and statistical analyses of both bacterial counts after different periods (24, 48 and 72 hours) and also the gradients of the growth curves were conducted using SPSS 16 software at temperatures of 25 and 35°C. One-way analysis of variance (ANOVA) in combination with the Tukey test was used to do mean comparisons and the differences between mean values were significant at the 5% confidence level. Statistical significance between treatments and the control group was assessed by REST® (Relative expression software tool 2009). A significant difference was defined as a P value <0.05.

Results
Effects of LA5 and LC01 on Staphylococcus aureus Growth
The growth of Staphylococcus aureus in either the presence or absence of Lactobacillus strains LC01 and LA5 was monitored at both 25 and 35°C. Growth curves of S. aureus shown in Figure 1 indicate that after 24 hours of incubation until the end of the experiment the titre of S. aureus in the control group was significantly greater than the corresponding titres in either of the other treatments (P<0.001) at 25°C. In addition, the titre measured in co-culture with LA5 during the last 48 hours of the experiment was significantly lower than that of the co-culture with LC01 (P<0.05). The comparison of linear gradient of S. aureus growth at 25°C showed that the increasing trend of bacteria number (gradient at 0.052±0.01) was significantly higher at control group in comparison with groups containing LC01 (gradient at 0.028±0.003) (P=0.018) and also with groups containing LA5 (0.006±0.004) (P<0.001); also the increasing trend was significantly higher in LC01 than LA5 (P=0.029).

The results presented in Figure 2 corresponding to S. aureus growth at 35°C show similar trends to those seen at 25°C. The titre of S. aureus was always greater in the control than in the LC01 and LA5 treated samples (P<0.001) and the LA5 treated sample showed significantly lower numbers of S. aureus compared with the LC01 sample during the final 48 hours of the experiment (P<0.05).

Enterotoxin Assay
Enterotoxin production by S. aureus is shown in Table 1. Whilst LC01 inhibited the production of SEA at both temperatures, LA5 showed no observable inhibitory effect.

![Figure 1](image1.png)

**Figure 1.** Logarithmic Plot of Staphylococcus aureus Growth in the Presence of LC01 and LA5 Bacteria vs the Control Culture at 25°C.

![Figure 2](image2.png)

**Figure 2.** Logarithmic Plot of Staphylococcus aureus Growth in the Presence of LC01 and LA5 Bacteria vs the Control Culture at 35°C.

![Figure 3](image3.png)

**Figure 3.** Relative sea Gene Expression of Staphylococcus aureus Co-cultured With Commercial Strains of Lactobacillus at 25 and 35°C.
Gene Expression Assay

The analysis of sea gene expression by S. aureus co-cultured in the presence of commercial Lactobacillus strains at 2 different temperatures is presented in Figure 3. All co-cultures showed down-regulation of the target gene compared with the control group. However, this reduction was dependent on both temperature and the presence of Lactobacillus strain. For example, at 25°C the transcriptional levels of sea in S. aureus co-cultured with LA5 and LC01 were respectively 6.42 and 10.31 fold lower than the control group, whereas at 35°C this reduction was 2.83 fold for LC01 and that for LA5 was not significant (P > 0.05). The greatest down-regulation of sea was observed in co-incubation of S. aureus with LC01 at 25°C.

Discussion

Staphylococcus aureus can grow across a wide range of environmental conditions and is a frequent contaminant of food. This contamination can originate from raw materials (eg, mastitic milk), from the processing plant environment (eg, biofilms on processing surfaces) or from handlers activity during food preparation and manipulation. The frequency of S. aureus contamination and the effect of staphylococcal food poisoning incidences on public health justify the interest taken by the scientific community and agro-food industries in combating this problem. A great deal of attention has also recently been given to certain foods as potential vehicles for antimicrobial compounds. Such foods have become important health care sectors in most countries, and among them, dairy products containing LAB such as Lactobacilli are of particular relevance (12). LAB can produce antimicrobial substances with the capacity to inhibit the growth of pathogens and spoilage microorganisms. In this study the effects of two commercial Lactobacillus strains (L. acidophilus LA5 and L. casei 01), isolated from some fermented dairy products, and were studied on the growth, enterotoxin production and sea gene expression in S. aureus under co-culture conditions.

The inhibitory potential of LAB on S. aureus growth has been described in various studies.13-15 The results of the present study indicate that at 25°C the inhibition of both S. aureus growth and sea expression is greater than that at 35°C in both Lactobacillus co-cultures. Samehashima et al and Gonzalez-Fandos et al indicated that S. aureus growth inhibition varied depending on the temperature.16,17 Troller and Frazier reported that maximum inhibition of S. aureus growth, in association with other organisms, occurred at temperatures of 20 to 25°C, supporting our results.18 Other previous reports also suggest that growth of S. aureus is generally inhibited to a greater degree at temperatures lower than 30°C when cultured with other organisms.13,19

A number of propositions have been made to explain the mechanism of inhibition of S. aureus growth by LAB, including the production of bacteriocins and hydrogen peroxide.13,19 competition for nutrients, and acidification.3 LAB are known to produce a range of antimicrobial compounds including lactic acid, acetic acid, formic acid, phenyllactic acid, caproic acid, organic acids, ethanol, hydrogen peroxide, diacetyl, bacteriocins, reuterin, reutericyclin and bactericidal proteins.20,21 It was previously demonstrated that L. acidophilus LA5 secreted molecules influenced on pathogens as E. coli O157 secretion system.22 Wang et al detected the down regulation of important virulence-related gene expression after S. aureus, E. coli O157 and Shigella flexneri were grown in medium supplemented with biologically active fractions of L. acidophilus La-5 CFSM compared with the same bacteria grown in the same medium without the addition of La-5 fractions. They also found that the count of S. aureus decreased after mixing with L. acidophilus LA5 and L. acidophilus ATCC 4356 over 1 hour.23 Dicks and Botes24 had reported that hydrogen peroxide produced by some strains of Lactobacilli, effectively inhibits S. aureus, and L. acidophilus isolated from humans due to production of bacteriocin and non-bacteriocin antimicrobial substances which are active ( both in vitro and in vivo) tests against gram-positive and gram-negative pathogens.24 Tomioka et al has investigated the effect of ofloxacin combined with L. casei against Mycobacterium fortuitum induced infection in mice. They found a marked delay in the incidence of spinning disease and an increase in the rate of elimination of organisms from the kidneys.25 Among the virulence factors produced by S. aureus, enterotoxins are the main health threat in foodstuffs. In the present study, the effect of Lactobacillus strains on the level of sea gene expression was investigated by RT-PCR given the latter’s increased sensitivity and specificity compared to the conventional method.4,26 RT-PCR has been used previously to study S. aureus SE gene expression.27 Our data show that S. aureus co-culture with L. casei 01 resulted in the greatest level of down-regulation of sea gene expression at 25°C compared with L. acidophilus (LA5). At 35°C, L. acidophilus (LA5) was ineffective in inhibiting SEA production by S. aureus whilst co-culture with L. casei 01 showed weak sea down-regulation. Molecular approaches including transcriptomic ones are promising for increasing our

### Table 1. Comparison of Staphylococcus aureus Enterotoxin Production in the Mixed Cultures with Commercial Strains of Lactobacillus Incubated at 25 and 35°C After 72 Hours

<table>
<thead>
<tr>
<th>Incubation Temperature</th>
<th>Control</th>
<th>LA5</th>
<th>Lactobacillus casei 01</th>
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<tbody>
<tr>
<td></td>
<td>SEA</td>
<td>SEC</td>
<td>SEE</td>
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<tr>
<td>25°C</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>35°C</td>
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knowledge of the mechanism involved in inhibition of bacterial virulence factors by microbial interactions. In the present study, we showed by transcriptional expression analysis that lactobacillus isolates reduced sea expression in S. aureus. Very few gene expression studies have been carried out to evaluate the impact of probiotic bacteria on the gene expression of SEs as well as other exotoxins. Recently, Laughton et al reported that Lactobacillus ruteri produces a small-sized soluble compound which is able to interfere with the expression of an exotoxin gene in S. aureus. It was indicated that the impact of Lactococcus lactis on enterotoxin expression was enterotoxin type dependent; L. lactis strongly decreased the expression of sec and sel, while slightly favored the expression of sea. As it is observed from the results of the present study, L. acidophilus (LA5) had stronger inhibitory activity against the growth of S. aureus while L. casei 01 showed the greatest down regulation of sea. It is reported that the expression of virulence of an S. aureus strain may also be inhibited, even if no prevention of growth is occurred. The results of this study revealed that co-culture of S. aureus with 2 commercial Lactobacillus strains resulted in reductions in both growth (L. acidophilus was the most effective) and enterotoxin production (L. casei had the greatest effect). Furthermore RT-PCR analysis revealed down-regulation of the sea gene, a phenomenon which was also temperature and strain dependent. Our data therefore suggest that these isolates could be considered to be applied as natural inhibitory agents against bacterial growth and toxin production in order to maintain the quality and improve the safety of certain food products.

Authors’ Contributions
MP: Designing the study. Obtaining the samples and writing the manuscript; MA: drafting of the manuscript; AJJ: Conducting the statistical analyses.

Ethical Approval
All procedures performed in this study were in accordance with the ethical standards of the national research committee.

Conflict of Interest Disclosures
The authors declare that they have no conflict of interests.

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