

Expression of toxic shock syndrome toxin-1 gene of *Staphylococcus aureus* in milk: Proof of concept

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Abstract

The aim of this study was to assess the expression of a toxic shock syndrome toxin-1 (TSST-1) gene of *Staphylococcus aureus* in different types of milk, depending on inadequate temperature and storage conditions. Pasteurized and UHT milk were inoculated with monotoxic TSST-1 strain of *S. aureus* and growth kinetics was determined by the drop plate method using Baird-Parker agar medium in accordance with EN ISO 6888-1. The patterns of gene regulation were detected by quantitative reverse transcriptase PCR. Relative quantification method for statistical significance testing was used to detect significant gene expression responses. The results of experiments showed the dependence of the growth rate and consequent up-regulation of TSST-1 encoding gene on storage time-temperature and type of milk. In contaminated pasteurized and UHT milk kept at both 15 °C and 22 °C *S. aureus* significantly increased its virulent potential over time. This effect was a bit more emphasized in UHT milk serving as a “proof of concept”. Possible explanation could be a presence of lactic acid bacteria in pasteurized milk which is known to have down-regulatory effect on TSST-1 gene. Maintenance of the milk storage temperature below 8 °C and employment of microbiological control measures in hygienic practices, from milk producer through retailer and on to the consumer is of utmost importance to decrease risk of non-emetic staphylococcal poisoning.

Key words: *S. aureus*, toxic shock syndrome toxin-1, milk

Introduction

Staphylococcal food poisoning (SFP) is one of the most common foodborne illnesses resulting from ingestion of staphylococcal enterotoxins (SE) produced in food by enterotoxigenic strains of *Staphylococcus aureus*. Milk is considered to be a good substrate for *S. aureus* growth and enterotoxin production, and contaminated raw milk and dairy product have been often involved in SFP (Asao et al., 2003). Infected dairy animals suffering mastitis may contaminate bulk milk, Furthermore, human handlers, milking equipment, the environment, as

well as unhygienic udder and surrounding skin may be other possible sources of bulk milk contamination. From a food safety perspective, that is a concern because enterotoxigenic *S. aureus* may be a risk of SFP after consumption of raw milk products (Jorgensen et al., 2005). SFP is suspected when the symptoms including nausea, violent vomiting, abdominal cramps, and diarrhea affect the patients between 1 and 8 h after food consumption. To date, over 20 SEs have been characterized and many *S. aureus* strains can synthesize more than one type of toxins (Kerouanton et. al., 2007; Loncarevic

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et al., 2004; Tamarapu et al., 2001). These toxins are heat resistant low molecular weight proteins (MW 26.900-29.600), which means that their biological activity remains unchanged even after thermal processing of food (Martin et al., 2004; McKlauchlin et al., 1999). The five classic enterotoxins (SEA, SEB, SEC, SED, and SEE) are known to be responsible for 95 % of staphylococcal food poisoning cases and SEA and SED are most common enterotoxins recovered from food poisoning outbreaks, possibly because they can be produced in a wide range of growth conditions (aw and pH).

The remaining 5 % of outbreaks are associated to newly described SE's and TSST-1. It is difficult to obtain accurate estimates of the incidence of *S. aureus* intoxications because most cases are not reported.

Toxic shock syndrome (TSS), a relatively rare condition (0.06 cases per 100,000 people) caused by TSST-1 or more potent exotoxins is characterized by fever, rash, hypotension, constitutional symptoms, multi-organ failure and death (Saha et al., 1996; Thomas et al., 2007). Superantigen-producing *S. aureus* incorporated into the digestive tracts might cause enterocolitis (Watanabe et al., 2001) and persist for a long time in the intestinal microbiota of infants (Lindberg et al., 2000). Enteric TSST-1 activates systemic T cells and leads to a dysregulated, but exuberant immunological activity causing the remission and flare-up cycle of mucosal inflammation in patients with ulcerative colitis (Shiobara et al., 2007). TSST-1 producing strains of *S. aureus* are widespread in humans, animals and foods and such distribution may play significant role in the epidemiology of TSS, while the data on prevalence in the environment are currently very scarce.

Staphylococcal food poisoning is acquired by ingestion of preformed toxin and has been linked primarily to the SEs (Jablonski and Bohach, 1996). The main symptom - emesis - is often accompanied by nausea and abdominal cramping, but lacks of a fever and shock. It is still unclear why these two superantigenic toxins (SE and TSST-1) show different pathogenesis in infections and food poisoning induced by *S. aureus*. Research study conducted by Li et al. (2011) showed strong resistance of the superantigenic activity of TSST-1 to heat treatment, pepsin and trypsin digestion. Although TSST-1 was rapidly degraded to smaller fragments after

treatment with pepsin or trypsin, they retained significant superantigenic and lethal shock activities relative to those of SEA. These results indicated that the different resistance of SEA and TSST-1 participates in the different pathogenic activities of superantigenic toxins during food poisoning and TSS.

Although pasteurization kills *S. aureus* cells, heat-stable SEs and TSST-1 generally retain their biological activity (Evenson et al., 1988; Asao et al., 2003). Thus, because of the importance of these toxins in the public health and food sectors, an efficient screening to detect the prevalence of enterotoxic strains in foods is required. To cast some light on the TSST-1 toxin hazard in milk, we have conducted a research study aiming to develop "proof of concept" by comparing the expression of TSST-1 encoding gene of *S. aureus* in cases when different milk types were properly stored and as well as they suffered time-temperature abuse.

Material and methods

Growth modelling

Strain of *S. aureus* producing TSST-1 was obtained from the American Type Culture Collection (ATCC BAA-2094). Different types of milk that had previously tested negative for *S. aureus* have been inoculated with an average of 2.4×10^4 CFU mL⁻¹ of the above strain. The milk samples tested were pasteurized and UHT 1.6 % fat milk obtained from retail. Inoculated milk model samples were incubated at 8 °C (calibrator-untreated control sample), 15 °C and 22 °C (environmental temperature) to simulate inappropriate transport and storage conditions. Experiments were always conducted in parallel. Two groups of plates in each experiment were inoculated 12 hours apart to cover 24 hours. A 24-hour sampling interval was used for the extraction of TSST-1 encoding gene mRNA. The enumeration of *S. aureus* was performed at a 24-hour interval and the average values were calculated from the results of the parallel and repeated experiments. During the incubation, pH of the model samples was measured periodically (data not shown). Enumeration of *S. aureus* was performed using the Baird-Parker drop plate count method in accordance with EN ISO 6888-1 and the plates were cultured at 37 ± 1 °C for 24 ± 2 hours and 48 ± 2 hours.

RNA extraction with commercially available kit

Commercially available kit PureLink RNA Mini Kit (Invitrogen, USA) was used for RNA extraction, including lysis and silica membrane extraction according to the manufacturers' instructions and further optimized by using 25 mg/mL of lysozyme (Sigma, USA) for 60 min at 37 °C. This optimization resulted in a 2-4 fold increase in total RNA yield (data not shown).

Briefly, bacteria were resuspended in 100 µL RNase free water and transferred to a tube containing 0.4 g of acid-washed 150-212 mm silica beads (Sigma, USA), 400 µL Lysis buffer R (provided by the kit) and 400 µL 90 % phenol solution (Appli-Chem, Germany) vortexed for 20 seconds before using the BeadBeater cell disruptor (Biospec Products, USA) with setting 6.5 for 60 sec. The samples were cooled on ice and the beat-beading step repeated twice. Afterwards, samples were centrifuged for 5 min at 16,000 × g and supernatants are mixed with equal volume of 100 % ethanol (Sigma, USA). The samples (including any remaining precipitate) were transferred to the silica columns and centrifuged for 15 sec at 13,000 × g at room temperature (RT). Columns were washed with 700 µL of Wash buffer and centrifuged for 15 sec at 13,000 × g at RT. Second washing stage is 500 µL of Wash buffer II with the same centrifugation protocol. Finally, RNA was recovered in 40 µL of RNase-free water, incubated for 1 min and centrifuged for 1 min at 12,000 × g. All steps were done at room temperature, except where otherwise noticed.

DNase treatment

Genomic DNA was digested with RQ DNase I (Promega, USA). Briefly, 3 µL of DNase I and 3 µL of reaction buffer were added to the RNA sample and incubated at 37°C for 30 min. DNase I enzyme was inactivated with 5 µL of 25 mM EDTA, incubated for 10 min at 65 °C.

RNA quality determination

The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop 1000 (Thermo Scientific). Three independent measurements of the same sample were performed. The absorbance ratio A260/A280 was used as an indicator of protein contamination and A260/A230 as an indicator of polysaccharide, phenol, and/or chaotropic salt contamination. The integrity of the total RNA was assessed by visualization of the 23S/16S banding pattern. Electrophoresis was performed on 1.5 % agarose gel at 80 V for 60 min. The ethidium bromide stained gel was visualized by GelDoc2000 (Bio-Rad, USA). RNA was stored at -80 °C for further use.

cDNA synthesis

cDNA synthesis was performed using commercial Super Script VILO (Invitrogen, USA) kit, according to manufacturer's instructions. The same amount of total RNA (500 ng/20 µL) was reverse transcribed in two reaction volumes: 20 µL, as described by the manufacturer, and 10 µL. To determine the possibility of genomic DNA carry-over, control reactions were performed under the same conditions but lacking the reverse transcriptase enzyme (no-RT control). All RNA extracted was absent of significant genomic DNA, as determined by an average cycle threshold difference of 18.5 ± 3.5, equivalent to a maximum quantification error of 0.0003 %.

Gene expression quantification

TSST-1 gene expression was determined by qPCR. Oligonucleotide primers for the detection of *ftsZ* (housekeeping gene) and TSST-1 encoding gene (*tst*) were designed using the Primer3 software having *S. aureus* ATCC BAA-2094 genome, as template (Table 1).

Table 1. Primers used in gene expression analysis

Gene	Primer sequence	Amplicon size
<i>ftsZ</i>	F: 5'-ATCCAAATCGGTGAAAAATTAACAC-3' R: 5'-CCATGTCTGCACCTTGGATTG-3'	121 bp
<i>tst</i>	F: 5'-TCATCAGCTAACTCAAATACATGGATT-3' R: 5'-TGTGGATCCGTCATTCATTGTT-3'	88 bp

qPCR analysis was performed using Maxima SYBR Green Master Mix (Fermentas, Lithuania). For transcripts detection, 2× SYBR Green I (Invitrogen, USA) was added to a standard PCR mix. The 20 µL reaction volume contained 2 µL of diluted cDNA or no-RT control, 10 pmol of each primer, 6 µL nuclease free H₂O, and 10 µL of the respective 2× master mix. Primer efficiencies were determined by the dilution method as well as performing a temperature gradient reaction from 50 to 65 °C. At 60 °C, both set of primers had the best and more similar efficiencies values. qPCR run was performed on a MX3005P (Agilent, USA) with the following cycle parameter: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 68 °C for 15 s. qPCR products were analyzed by melting curves for unspecific products or primer dimer formation. Relative fold increase of specific mRNA transcripts in milk comparing with planktonic cultures, was calculated using 2^{ΔCt} method, a variation of the Livak method, where 2 stands for the 100 % reaction efficiency (the reaction efficiency was determined experimentally and thus 100 % efficiency was replaced by the real efficiency) and ΔCt=Ct (housekeeping gene)-Ct (target gene). The data analysis was based on 3 independent experiments.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to identify conditions when *tst* gene was altered by at least a 2.0-fold increase or decrease in expression between groups. The numbers of significantly up- and down-regulated genes in each comparison group were identified by applying a P value of <0.05, together with fold change cut-offs of 2.0-fold decrease and 2.0-fold increase.

Results and discussion

To the extent of our knowledge, this is the first study to characterize TSST-1 encoding gene expression changes of *S. aureus* grown in the different milk types, stored at inappropriate temperatures during prolonged period, compared to milk contaminated with the *S. aureus* and held at 8 °C. This temperature has been considered as a set off temperature, since deviations from it will result in an increased risk of microbiological hazards.

Over the past decades TSST-1 from *S. aureus* has been considered as the most important cause of TSS in humans. Therefore, the evaluation of the presence of TSST-1 producing *S. aureus*, as well as *S. aureus* pathogenic activity under different storage conditions assessing the combination of virulence genes is of great importance both in human and in veterinary medicine (Zecconi et al., 2006; Piccinini et al., 2009). Highly contaminated milk with *tst*-positive strain of *S. aureus* is a source of contamination of dairy products during processing, because staphylococcal toxins are highly heat resistant and survive after the commercially relevant heat treatment process (Le Loir et al., 2003). The main sources of milk contamination with high number of *S. aureus* are: i) animals, when cows contaminate milk during milking via infected udder; ii) unhygienic milking procedures, unsanitary equipment and inadequate storage conditions; iii) humans (handlers contaminate milk via manual contact or via respiratory tract by coughing and sneezing), when contamination occurs after heat treatment of the milk (Le Loir et al., 2003; Chye et al., 2004). Hence, it is highly important to quickly decrease the temperature of raw milk below 8 °C and to achieve the best storage conditions to keep milk fresh for as long as possible, but no longer than necessary. Also is required to respect the principles of good hygiene practice and good manufacturing practice (Chye et al., 2004).

In general, results from our experiments indicated that the *S. aureus* counts, as well as relative expression of *tst* gene increased in both pasteurized and UHT milk incubated at different temperatures during storage period.

In pasteurized milk inoculated with the *tst*-positive strain of *S. aureus* and stored at 15 °C for 72 hours, an increase of *S. aureus* count from 4.36 to 8.38 log₁₀ cfu mL⁻¹ was observed, as shown in Figure 1. During the same period, expression of *tst* gene was down-regulated by 0.67-fold after initial 24 hours of incubation (P=0.643), but significantly up-regulated within the next 24 hours, reaching 2.85-fold (P=0.012) increase after 48 hours of incubation (Figure 2). By the end of the Day 3, gene expression was still significantly up-regulated compared to milk stored at 8 °C, at the level 2.46-fold of increase (P=0.021). In inoculated UHT milk stored at 15 °C, similar growth pattern was observed,

increasing from 4.36 log₁₀ cfu mL⁻¹ (0 hours) to 8.48 log₁₀ cfu mL⁻¹ (72 hours). However, there was no downregulation recorded during incubation, but rather an insignificant increase of 1.36-fold (P=0.178) after 24 hours which continuously increased to 2.81-fold after 48 hours (P=0.015), and reaching plateau value of 2.73-fold increase after 72 hours (P=0.015).

As expected, growth pattern of *tst*-positive strain of *S. aureus* in pasteurized milk stored at 22 °C dramatically changed compared to pattern recorded at 15 °C (Figure 3). Namely, no lag phase was observed and growth kinetics increased exponentially reaching 7.55 log₁₀ cfu mL⁻¹ just after 24 hours and

reaching plateau of 8.28 and 8.41 log₁₀ cfu mL⁻¹ after 48 and 72 hours, respectively. At this storage temperature, *tst* gene relative expression increased to 1.83-fold (P=0.062) after 24 hours (compared to milk stored at 8 °C) and reached 3.18-, and 3.36-fold increase in 48 and 72 hours, respectively (P=0.002). In UHT milk stored at 22 °C, growth was somewhat similar, reaching 7.73 log₁₀ cfu mL⁻¹ just after 24 hours and reaching plateau of 8.32 and 9.00 log₁₀ cfu mL⁻¹ after 48 and 72 hours, respectively. *tst* gene relative expression increased to 2.57-fold (P=0.022) after 24 hours (compared to milk stored at 8 °C) and reached 2.74-, and 2.93-fold increase in 48 i.e. 72 hours, respectively (P=0.014) (Figure 4).

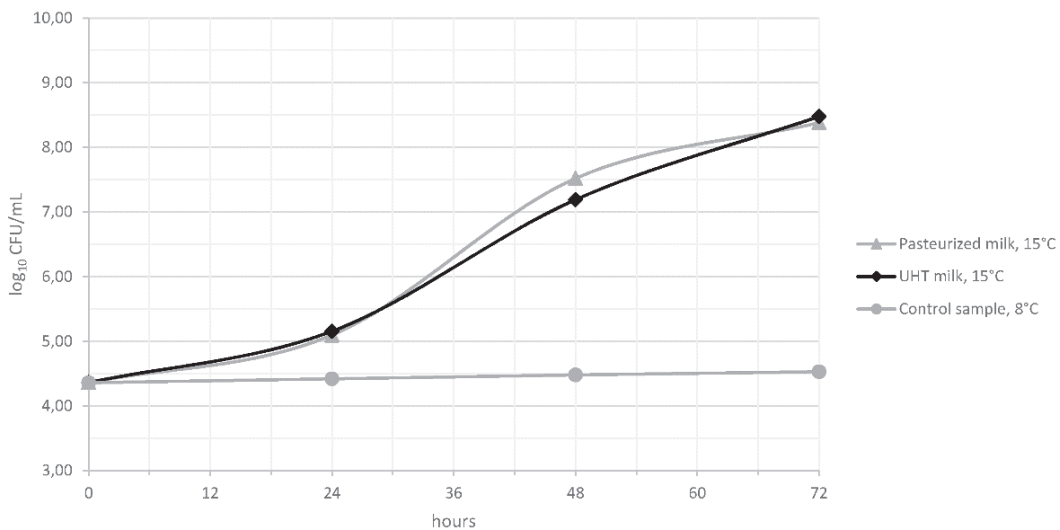


Figure 1. Growth kinetics of TSST1 positive *S. aureus* at 15 °C

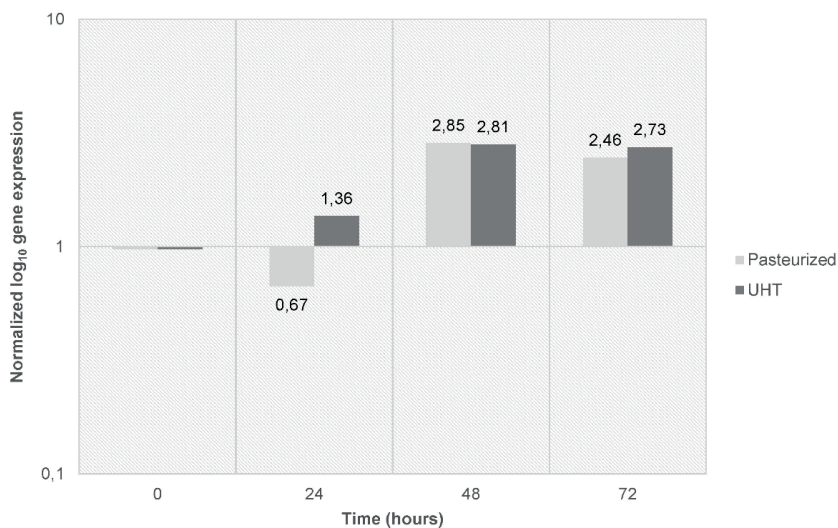


Figure 2. *tst* gene expression at 15 °C

Due to rather scarce data on potential risk of TSST-1 positive *S. aureus* in milk and dairy products, establishing incidence of this pathogen in milk is of critical importance. A comprehensive research study was conducted in 2015 by Vitale et al., who showed that from a total of 102 isolated *S. aureus* strains in a screening of 971 various food products, 89.2 % were originated from dairy products, while the subsequent molecular analysis of the toxin genes of 102 isolated *S. aureus* strains, showed that more than 42 % harbored the *tst* gene, whereby about 80 % of the strains that carried the *tst* gene (35/43) were isolated from dairy products. However, some authors reported lower occurrence of TSST-1 *S. aure-*

us in food samples, i.e. 13.5 % of the samples tested in Korea (Oh et al., 2007) and 12% in Iran (Dallal et al., 2010). Srinivasan et al. (2006) investigated the prevalence of 16 enterotoxin genes (SEA-SEE and SEG-SEQ) and toxic shock syndrome toxin gene (TSST-1) of *Staphylococcus aureus* isolated from milk of cows with mastitis. From a total of 78 *S. aureus* examined, TSST-1 gene was detected in 20 (25.6 %) isolates and was always found in combination with other enterotoxin genes. They considered that the high prevalence of enterotoxin genes and TSST-1 in *S. aureus* may be important as it is relevant to udder pathogenicity and food hygiene. In contrast, Arcuri et al. (2010) have examined 291

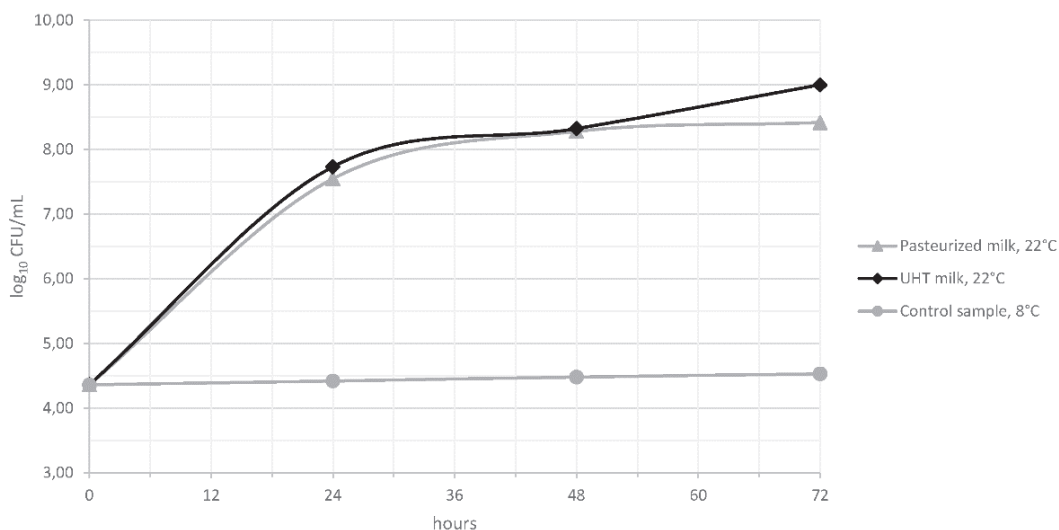


Figure 3. Growth kinetics of TSST1 positive *S. aureus* at 22 °C

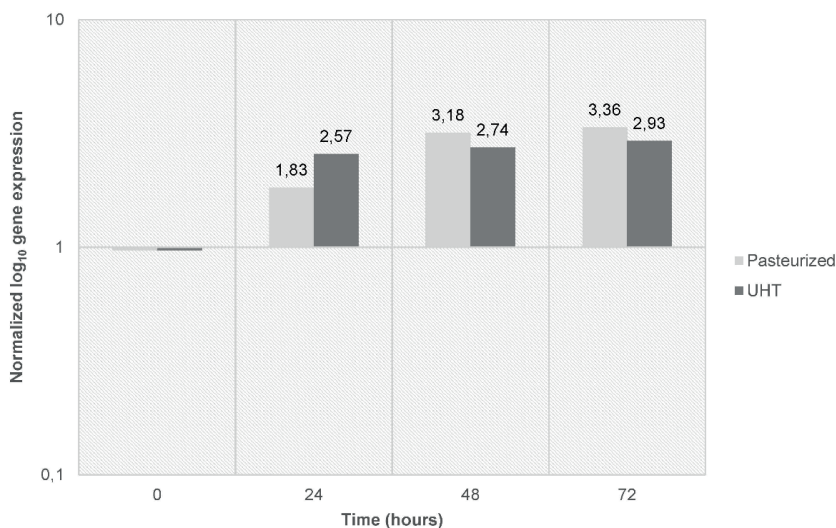


Figure 4. *tst* gene expression at 22 °C

Staphylococcus aureus isolates from mastitic cow's milk, bulk tank milk, and Minas fresh cheese for staphylococcal enterotoxin genes and TSST-1 by PCR assay, and they found the *tst-1* gene of *S. aureus* in only eight isolates, but none from mastitic cow's milk.

One of the possible explanations why *tst* gene was initially down-regulated in pasteurized milk is presence of lactic acid bacteria. Almost all of the staphylococcal super-antigens are encoded on variable genetic DNA elements, with the exception of SE-1 X, which is encoded on the core chromosome. TSST-1 is encoded by genes on *S. aureus* pathogenicity islands (SaPIs) (McCormick et al., 2001). Like other exo-proteins and cell surface virulence factors, TSST-1 staphylococcal super-antigen is controlled by global regulatory systems designated as accessory gene regulator (*agr*). It was not our intent to discuss these *agr* regulatory elements in detail. Readers are encouraged to read important reviews that discuss global regulatory pathways in detail (Novick, 2003; Pragman and Schlievert, 2004). However, Nouaille et al (2014) established the ability of *Lactococcus lactis*, a lactic acid bacterium widely used in the dairy industry, to down-regulate a major staphylococcal virulence regulator, the *agr* system, and, as a consequence, *agr*-controlled toxins. The *agr* system relies on at least two general properties of LAB: their ability to decrease both pH (Cretenet et al., 2011) and redox potential via oxygen-independent mechanisms (Nouaille et al., 2014).

Milk is highly vulnerable to contamination with *S. aureus*, especially at higher temperature during prolonged storage, so the aim of this study was to determine the expression of TSST-1 encoding gene of *S. aureus* when milk was stored under different temperatures during prolonged time. The incubation of milk at 15 °C and 22 °C (room temperature) was used to simulate inappropriate transport and storage conditions. In this study, the number of *S. aureus* in both pasteurized and UHT milk stored at 8 °C remained unchanged during the entire storage period. In contrast, the increase in storage temperature and storage time of both, pasteurized and UHT milk, resulted in increased number of *S. aureus*, as also observed by Janštová et al. (2012). The number

of *S. aureus* of pasteurized and UHT milk cultured at 15 °C and 22 °C for 24 h, exceeded the threshold of 10^5 CFU mL⁻¹, which represents the risk for enterotoxin production (Duquenne et. al., 2010). Also, some authors (Fujikawa and Morozumi, 2006; Medve'ová et al., 2009) reported an increase in the number of *S. aureus* at different incubation temperatures of milk, and consequently the increase in the virulence of the pathogen. *S. aureus* is able to grow in a wide range of temperatures from 7 °C to 48.5 °C, with the optimum growth being observed at 30 °C to 37 °C, while the optimal temperature for enterotoxin production is between 10 °C and 46 °C (Schmitt et al., 1990). In our study, the highest *S. aureus* counts were recorded for the strain producing TSST-1 toxin when cultured in pasteurized and UHT milk at 22 °C.

Conclusions

Given that milk is derived from animals, it inherently carries the risk of being contaminated with pathogens from its source (cattle, goats, sheep, and the farm environment). Although TSST-1 is usually not suspected to cause staphylococcal enterotoxicosis, due to lack of classical symptoms such as "projectile vomiting". Results obtained in this "proof of concept" model showed that abuse of storage temperature in combination with prolonged storage time of contaminated milk may significantly increase TSST-1 health hazard. The key factor in the prevention of milk-borne disease is consumer avoidance of raw milk consumption and implementation of good hygiene practices and effective monitoring from production through the delivery chain, retailer and to the consumer, especially maintaining the storage temperature below 8 °C.

Acknowledgments

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Ekspresija gena za toksični šok sindrom toksin 1 Staphylococcus aureus u mlijeku - dokaz koncepta

Sažetak

Cilj ovog istraživanja bio je ispitati ekspresiju gena za sintezu toksina 1 toksičnog šok sindroma (TSST-1) *Staphylococcus aureus* u različitim vrstama mlijeka držanim u neadekvatnim temperaturnim uvjetima. Pasterizirano i UHT mlijeko inokulirano je monotoksičnim TSST-1 pozitivnim sojem *S. aureus* i držano pri 15 °C i 22 °C tijekom 72 sata. Kinetika rasta određena je *drop plate* metodom korištenjem Baird-Parker agara sukladno EN ISO 6888-1. Regulacija gena za TSST-1 određena je lančanom reakcijom polimerazom uz reverznu transkripciju u realnom vremenu. Za određivanje promjena u ekspresiji gena, korištena je metoda relativne kvantifikacije. Rezultati ispitivanja pokazali su da su stopa rasta *S. aureus* kao i posljedična regulacija gena za sintezu TSST-1 ovisni o intenzitetu temperature, a djelomično i o vrsti mlijeka. Povećana regulacija ovog gena utvrđena je pri obje temperature, ali stopa porasta virulentnog potencijala bila je znatnije izražena u UHT mlijeku držanom pri 22 °C koje je služilo kao model u dokazu koncepta. Jedan od mogućih razloga za niži virulentni potencijal u pasteriziranom mlijeku bilo je prisustvo bakterija mliječne kiseline za koje se zna da imaju represivni utjecaj na ekspresiju gena za sintezu TSST-1. Održavanje temperature čuvanja mlijeka ispod 8 °C i primjena kontrolnih mikrobioloških mjera u higijeni mlijeka od proizvođača, preko maloprodaje do krajnjeg potrošača, od kritičnog je značaja za smanjenje rizika nastanka stafilokoknih trovanja bez pojave povraćanja.

Ključne riječi: *S. aureus*, toksin 1 toksičnog šok sindroma, mlijeko

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