



## STAPHYLOCOCCUS AUREUS AND ENTEROTOXIN A RELATIVE GENE EXPRESSION IN BEEF MEAT AFTER LACTIC ACID TREATMENT AND STORAGE AT DIFFERENT TEMPERATURES

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### Summary

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The *Staphylococcus aureus* enterotoxin A (SEA) is the toxin mostly involved in *Staphylococcus aureus* (*S. aureus*) food poisoning. In this study, the effect of different lactic acid (LA) concentrations (LA 1% and 2%) and temperatures (4 °C, 25 °C, and 37 °C) on *S. aureus* growth and relative *sea* expression in fresh meat cuts were studied. Real-Time RT-PCR used to determine the relative *sea* expression. Fresh meat cuts were inoculated with 10<sup>5</sup> CFU/g of *S. aureus* producing enterotoxin A. *S. aureus* growth and relative *sea* expression were regularly tested for 48 hours. The growth of *S. aureus* was decreased by one log CFU/g than control sample using 1% LA and 2% LA (5.32 ± 3.76 log CFU/g, 4.38 ± 3.00 log CFU/g and 4.54 ± 3.18 log CFU/g respectively) at zero time. Relative expression of the *sea* gene in both LA concentrations was lower than control. Moreover, both lactic acid concentrations had effect on relative *sea* gene expression at all examined hours, especially at 4 °C compared to control samples. The higher the lactic acid concentration, the lower the *S. aureus* enterotoxin A relative expression was.

**Key words:** lactic acid, RT-PCR, SEA, *sea* gene expression, *Staphylococcus aureus*, SYBR Green I

### INTRODUCTION

One of the commonest food-borne illnesses worldwide is *Staphylococcus* food poisoning (SFP) through ingestion of food contaminated with *Staphylococcus* enterotoxins (SETs) (Hennekinne *et al.*, 2012) produced in food during *S. aureus* growth

(Kérouanton *et al.*, 2007). *S. aureus* food poisoning is often caused when a food handler contaminates food products such as desserts, salads, or baked goods that are served or stored at room or refrigerator temperature (Anonymous, 1997). *S. au-*

*reus* belongs to family Staphylococcaceae and is the etiologic agent predominantly associated with staphylococcal food poisoning. It is Gram-positive, non-motile, catalase-positive, small, spherical bacterium (coccus) which appears in pairs, short chains or bunches of grape-like clusters (Lampel *et al.*, 2012). Recently, 21 different SETs alphabetically named as enterotoxin A (SEA), B (SEB), C (SEC) etc. and the respectively encoding genes *sea*, *seb*, *sec*, *sed*, *see*, etc. were identified (Schelin *et al.*, 2011). Nevertheless, staphylococcal enterotoxin A (SEA) and B (SEB) are the most frequently reported in SFP (Cha *et al.*, 2006; K erouanton *et al.*, 2007). SETs are highly heat-stable as well as proteolytic enzymes resistant enterotoxins (Lampel *et al.*, 2012). *S. aureus* is one of the most resistant non-spore forming human pathogens which survives much longer in a dry state. It is ubiquitous and can be found in soil, water, and air (Lampel *et al.*, 2012). However, the mucous membranes of the nasopharynx and human and animal skin are the primary habitats of *S. aureus* (Hatakka *et al.*, 2000). *S. aureus* produces a wide variety of toxins apart staphylococcal enterotoxins, including virulence factors as various enzymes, cytotoxins, pyrogenic exotoxins and toxic shock syndrome toxin 1 (TSST-1) besides exfoliative toxins (Novick *et al.*, 2001).

Nowadays, the evaluation of the safety of food products for human consumption occurs through viable cell counts; however, investigation and identification of the conditions that prevent or stimulate enterotoxin production and formation in food through measuring the enterotoxin levels in case of SFP is a more accurate way to determine the food safety (Wallin-Carlquist *et al.*, 2010a).

Acidification, especially using weak organic acids like lactic acid is commonly

used in the food industry to control spoilage and food poisoning bacteria. Lactic acid has a long history as a food preservative due to its general antimicrobial activity.

Reverse transcription polymerase chain reaction (RT-PCR) is highly sensitive and is the best technique to analyse mRNA expression derived from various sources. In general, there are two methods for achieving gene expression: the first is the relative quantification real-time RT-PCR using SYBR Green I fluorescence dye based on the relative expression of a target gene transcript versus a reference gene transcript and the second, absolute quantification based on an internal or an external calibration curve (Pfaffl, 2001; Chini *et al.*, 2007). The relative RT-PCR determines the expression level in comparison with a reference sample as well as is based on the expression level of a target gene in comparison with a housekeeping gene (Pfaffl, 2001; 2004; Chini *et al.*, 2007). However, the absolute RT-PCR depends on the calibration curve without need from neither reference nor comparison and it may need a more complex and time-consuming quantification for calculation of the calibration curve. The relative expression ratio is calculated only from the RT-PCR efficiencies and the crossing point deviation of an unknown sample versus control (Pfaffl, 2001; 2004; M arta, 2012).

Consequently, relative quantification method does not require either standards with known concentrations nor calibration curve as all samples are expressed as a ratio towards the housekeeping gene beside it is adequate for investigating physiological changes in gene expression levels (Pfaffl, 2004; Chini *et al.*, 2007).

All nucleated cell types contain housekeeping genes which are necessary for

basic cell survival (Pfaffl, 2001). Although numerous studies have shown that the housekeeping genes are regulated and vary under experimental conditions, the mRNA is stable and secure in various tissues even under experimental treatments (Zhang, 1992; Bhatia *et al.*, 1994; Marten *et al.*, 1994; Thellin *et al.*, 1999). SYBR Green I fluorescence dye is used as the simplest detection technique for newly synthesised PCR products in real-time PCR (Pfaffl, 2001; Márta, 2012).

The objective of this study was to investigate the effect of two lactic acid concentrations on *Staphylococcus aureus* growth and relative *sea* expression by comparison of the distinct cycle determined using crossing point at a constant level of fluorescence with kinetic PCR efficiency correction, in fresh beef meat during storage at 4 °C, 25 °C and 37 °C.

## MATERIALS AND METHODS

### *Bacterial strains and culture preparation*

*S. aureus* MK158948 (GenBank accession No.), isolated from milk products collected from retail markets in Gharbia governorate, Egypt, was obtained from the Reference Lab for Examination of Food of Animal Origin, Animal Health Research Institute (AHRI), Egypt.

The working culture of *S. aureus* (MK158948) was prepared from stock solution stored at -80 °C in cryovials (Copan, CRYOBANK™ Bacterial Cultures Freezing System; containing 25 colour-coded chemically treated ceramic beads with affinity to attach the preserved microorganisms suspended in a special cryogenic preserving solution). One ceramic bead was inoculated in brain heart infusion broth (Oxoid, CM 1135) and incubated overnight at 37 °C for 24 hours prior to the experiments.

### *Meat sample preparation*

Under complete aseptic conditions, fresh meat (free from *S. aureus* and its toxins) was cut into small cubic pieces (surface area of ~16 cm<sup>2</sup> and weight ~15 g) using sterile scissors. For each storage temperature, 1,500 g of meat pieces were dipped into an equal volume of overnight working culture inoculum (the *S. aureus* suspension developed with the McFarland standard and the equivalence corresponds to 3.0×10<sup>8</sup> CFU/mL) (Bogdanovičová *et al.*, 2017), then kept in the refrigerator at 4 °C for 20 min for bacterial attachment (Zeaki *et al.*, 2014) and let to dry for 15 min at Laminar Air Flow (model Nu. Aire serial no. 77265).

Then all inoculated samples were divided into three groups: (i) control group: 500 g meat pieces without any treatment while dipped in sterile deionised lactic acid free water in correspondence to lactic acid dipping treatment; (ii) group LA 1%: 500 g of meat pieces dipped into 500 mL 1% lactic acid (Research-lab Fine Chem Industries) and let to dry for 15 min at Laminar Air Flow, and (iii) group LA 2%: 500 g of meat pieces dipped into 2% lactic acid and let to dry for 15 min at Laminar Air Flow.

Aseptically, each group was packaged in sterile polyethylene bags and sealed using an impulse heat-sealing machine (Model PFS-300).

### *Sampling*

Samples were taken directly from each group after inoculation (zero time) for enumeration of *S. aureus* and relative *sea* expression and then each group was stored at three different temperatures (4 °C, 25 °C, and 37 °C). Samples were taken from each group every hour up to and including hour 8, then after 24 and 48 hours for *S. aureus* enumeration. Samples

were collected after 2, 4, 6, 8, 24 and 48 hours for relative *sea* expression (Wallin-Carlquist *et al.*, 2010b). Three replicate samples were collected from each group at each sampling time. The experiment was performed in three independent replicates.

#### *Viable counts determination*

According to Anonymous (2016), 10 g of each sample were homogenised using stomacher (Seward stomacher 80 Biomaster, England) with 90 mL of sterile Butterfield's phosphate-buffered dilution water (LOBA Chemie, CAS no. 7778-77-0) obtaining 1:10 dilution. Tenfold serial dilutions of stomacher liquid in 0.1% sterile peptone water (LAB M, LAB 104, lot 143257/110) 1 mL sample suspension) were aseptically transferred to the dried surface of 3 Baird Parker agar plates (Oxoid, CM0275) at 0.4 mL, 0.3 mL, and 0.3 mL and the inoculum spread using sterile bent glass streaking rod. The plates were retained in upright position until the inoculum was absorbed by the agar and then inverted and incubated at 35 °C for 48 hours to determine the total count of *S. aureus* (by summation of *S. aureus* in the three BP plates and multiplication by the sample dilution factor).

#### *Relative sea gene expression*

Extraction of mRNA was done using EasyPure<sup>®</sup>RNA kit, Transbionovo following its manufacturer's instructions: 200 µL of sample (from stomached liquid prepared in 2.3.1.) was mixed with 20 µL of Proteinase K, then 200 µL of Binding Buffer 5 with carrier RNA was added, vortexed for 15 s, and incubated at 56 °C for 15 min. Then 250 µL of 100% ethanol was added to the sample, mixed by vortexing for 15 s and incubated at room temperature (15–25 °C) for 15 min. The

entire content was transferred to a spin column and centrifuged at 12,000×g for 1 min with discarding flow through; 500 µL washing buffer was added, followed by centrifugation at 12,000×g for 1 min, with discarding the flow-through liquid. This step was repeated once, then centrifuged at 12,000×g for 1 min.

Finally, the spin column was placed into a clean RNase-free 1.5 mL microcentrifuge tube with 20 µL RNase-free water to the center of the column, incubated at room temperature for 1 min and centrifuged at 12,000×g for 1 min. The eluted RNA was stored at –80 °C until use.

The RNA extract was mixed with a master mixture of reverse transcriptase RT buffer and RT primer mix containing SYBR<sup>®</sup> Green I dye and incubated at 42 °C for 15 min, followed by further incubation at 85 °C for 5 s to inactivate the reverse transcriptase.

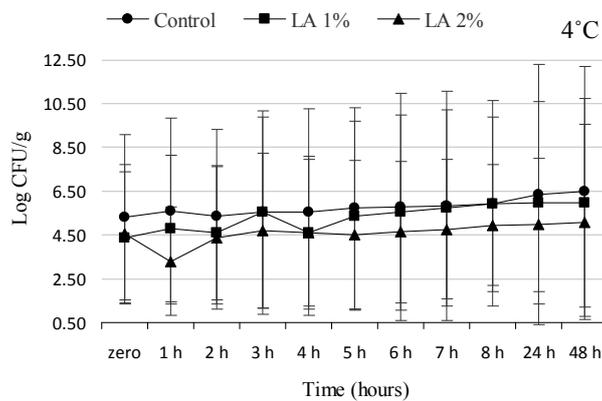
The synthesised oligonucleotide primers (Startechnology) used in this study are illustrated in Table 1. The PCR protocol comprised initial denaturation at 95 °C for 1 min, followed by 45 cycles of denaturation at 95 °C for 0 s (Wallin-Carlquist *et al.*, 2010b) primer annealing at 46 °C (*sea*) or 48 °C (rRNA) for 5 s and extension at 72 °C for 25 s, with a single fluorescence measurement at the end of the extension step. The real-time PCR data acquisition and analysis were performed using Analysis computer system, V 2.2.2 software (AB Applied Biosystems). Calculation of relative *sea* expression was determined using the following equation described by Pfaffl (2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}}(\text{control} - \text{sample})}}$$

where: Ratio is a relative expression ratio between the target and the reference

**Table 1.** Sequences of primers used for PCR in the present study (Wallin-Carlquist *et al.* 2010b).

Target gene	Primer	Nucleotide sequence (5'→3')
<i>sea</i>	ESA-1	ACGATCAATTTTACAGC
	ToxA reverse	CCGAAGGTTCTGTAGAAGT
<i>rrn</i>	rRNA forward	TGCGTGAGATGTTGGG
	rRNA reverse	ACTAGCGATTCCAGCTT



**Fig. 1.** Viable *S. aureus* counts (log CFU/mL) in beef meat either treated with 1% and 2% lactic acid or untreated at 4 °C. Values are expressed as the mean ± SD.

gene;  $E_{target}$  is the amplification efficiency (real-time PCR efficiency) of the target gene transcript;  $E_{ref}$  is the amplification efficiency (real-time PCR efficiency) of a reference gene transcript;  $\Delta CP_{target}$  is the CP deviation of control – the deviation of sample of the target gene transcript;  $\Delta CP_{ref}$  is the CP deviation of control – the deviation of sample of the reference gene transcript.

#### Statistical analysis

Statistical processing of *S. aureus* viable counts and the calculation of relative *sea* expression were performed using Microsoft Excel 2016. Data are given as mean ± standard deviation.

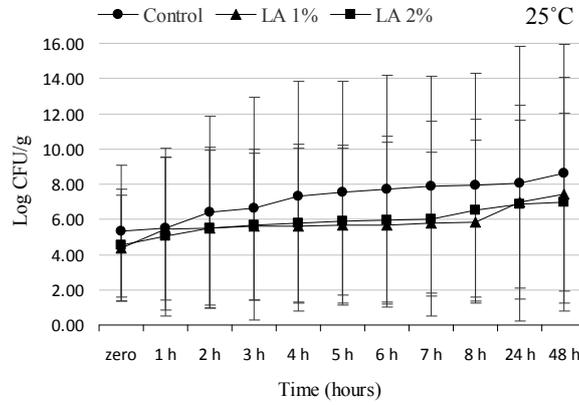
## RESULTS

### Viable counts

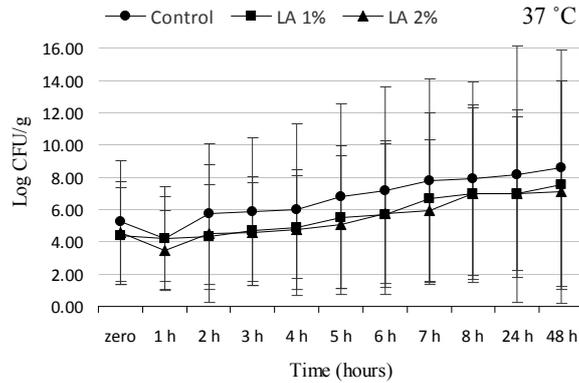
The effect of 1% and 2% LA on viable counts at 4 °C, 25 °C, 37 °C (Fig. 1–3) showed that the viable counts of the control group and after using LA 1% and 2% were  $5.32 \pm 3.76$  log CFU/g,  $4.38 \pm 3.00$  log CFU/g and  $4.54 \pm 3.18$  log CFU/g respectively at zero time. The viable counts of groups treated with 1% and 2% LA were lower at 1, 2, 3, 4, 5, 6, 7, 8, 24, 48 hours of storage at the three tested temperatures.

### Relative *sea* gene expression

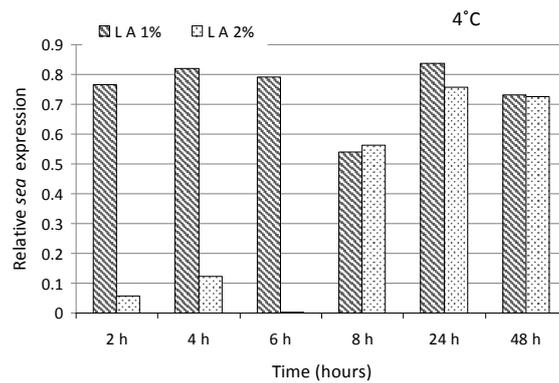
Results of relative expression of *sea* gene in LA 1% and LA 2% at 4 °C, 25 °C and 37 °C are shown in Fig. 4–6. It was found



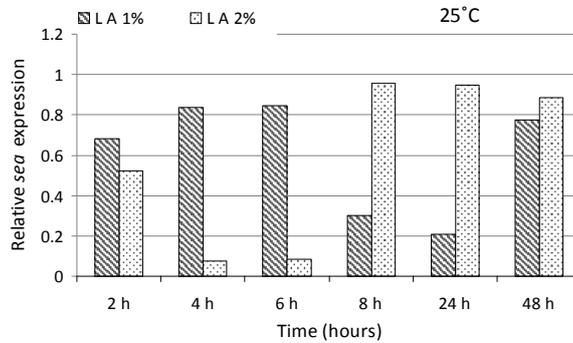
**Fig. 2.** Viable *S. aureus* counts (log CFU/mL) in beef meat either treated with 1% and 2% lactic acid or untreated at 25 °C. Values are expressed as the mean ± SD.



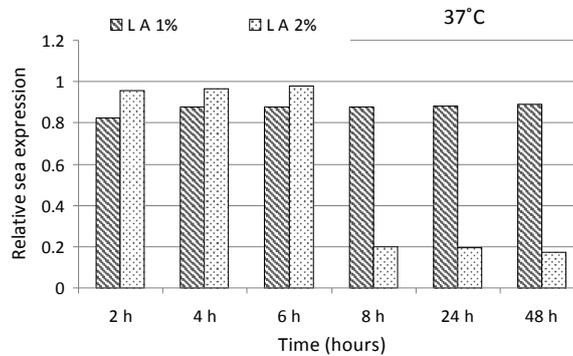
**Fig. 3.** Viable *S. aureus* counts (log CFU/mL) in beef meat either treated with 1% and 2% lactic acid or untreated at 37 °C. Values are expressed as the mean ± SD.



**Fig. 4.** Relative *sea* gene expression between the two concentrations LA (1%, 2%) compared using the rRNA housekeeping gene at 4 °C.



**Fig. 5.** Relative *sea* gene expression between the two concentrations LA (1%, 2%) compared using the rRNA housekeeping gene at 25 °C.



**Fig. 6.** Relative *sea* gene expression between the two concentrations LA (1%, 2%) compared using the rRNA housekeeping gene at 37°C.

that the relative expression of the *sea* gene in all treatments was decreased compared to control. At 4 °C, 2% LA decreased *sea* gene expression more significantly than LA 1% at all examined hours. At 25 °C, 2% LA decreased *sea* gene expression more at post-treatment hours 2, 4 and 6 hours than 1% LA, while the higher LA concentrations had more significant effect on *sea* gene expression at hours 8, 24 and 48. At 37 °C, the effect of 2% LA was much higher in decreasing *sea* gene expression than that of 1% LA at hours 2, 4 and 6, but not on hours 8, 24 and 48.

## DISCUSSION

This study investigated the potential of *S. aureus* growth and its *sea* gene expression at two LA concentrations acting as two pH levels of concentrations with a relatively high pKa (pKa is the negative base-10 logarithm of the acid dissociation constant Ka of a solution, pKa of lactic acid is 3.8) at three different temperatures (4 °C, 25 °C & 37 °C). There is a linear relationship between hydrogen ion concentration and the growth rate as well as LA concentration (Jamshidi *et al.*, 2008; Alibayov *et al.*, 2015; Ronaghi *et al.*, 2016).

In the study, the viable *S. aureus* counts for both LA treatments decreased at a higher extent at 4 °C than at 25 and 37 °C because *S. aureus* is a mesophilic bacterium and the temperature is the most important factor that controls its growth as well as pH and water activity (Lanciotti *et al.*, 2001; Fujikawa & Morozumi, 2006).

Alongside, temperature plays an important role to regulate the *S. aureus* growth and SEAs production level as lower temperature limits the microbial growth and the organism loses the ability to supply the maintenance requirements for growth (Min *et al.*, 2013). Probably the most important effect of temperature on the growth of a microorganism depends mainly on the intactness and good performance of the enzymes required for metabolism and that are present only within a relatively narrow temperature range (Jamshidi *et al.*, 2008).

LA has an inhibitory effect on *S. aureus* growth and enterotoxin A production as lactic acid stimulates the gradual formation of the electrochemical proton, which is important in the microorganism metabolism, the microorganism consumes more energy and energy deficiency affects its growth (Lin *et al.*, 2018).

LA concentrations causes variation in pH, water activity and moisture content besides the storage temperature whose increase also decreases the moisture content of the food products (Bingöl *et al.*, 2013).

Generally, bacteria require pH values between 4 and 8 for growth and survival (Erdoğan *et al.*, 2006) although water activity between 0.935 to 0.950 and pH 6.04 to 6.25 were considered optimal values for *S. aureus* growth and SAE production (Lin *et al.*, 2018).

Lactic acid and other weak organic acids are lipophilic, which enables acid

molecules to diffuse freely across the bacterial cell membrane. Once inside, the acid may dissociate and release protons that acidify the cytoplasm. Energy is diverted to maintain internal pH, and hence, growth is reduced or inhibited (Ronaghi *et al.*, 2016). However, the mechanisms of bacterial response to acidic environments are very complex and the tolerance varies widely between strains and species (Foster, 2000; Merrell & Camilli, 2002).

Expression of the *sea* gene decreased in both lactic acid concentrations compared to the control group, but the effect of 1% LA was more potent than that of 2% LA. This may support the observations of increased toxin production as the stress levels increased with high lactic acid concentration (Lovenklev *et al.*, 2004; Wallin-Carlquist *et al.*, 2010a). Similarly, it was suggested that more SEA was produced in acidic environments since it was found out that *sea* expression was slightly increased in the mild acidic environment of a cheese matrix and in the presence of *Lactococcus lactis* bacteria (Cretenet *et al.*, 2011). The observed pH change over time suggests that the lactic acid concentration, as well as other chemical compounds in the medium, may have changed during the incubation period. In response to lactic acid exposure, it has been reported that the pH gradually increased during *S. aureus* growth from pH 4.5 to 7.5 within 24 h due to ammonium accumulation and removal of acidic groups (Rode *et al.*, 2010).

## CONCLUSION

The results of this study indicated that both tested lactic acid concentrations (acidic pH) had an effect on *S. aureus* viable counts and *sea* gene relative expression besides supporting and ensuring

the safety of meat. There is a great need for rising up, developing and spreading the hygienic knowledge, attention and control measures where meat is handled and served as well as to promote good personal hygiene to reduce spreading of *S. aureus*. The storage temperature of fresh meat triggers the proliferation of *S. aureus* and enterotoxin production so cold storage of the meat at 4 °C is preferable. Real-time PCR using SYBR Green I fluorescence dye used in this study can be used as a rapid method for detection of *sea* mRNA molecules as it saves time and does not need complex techniques.

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