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Effect of Salt Ratios on Enterotoxin Production by *Staphylococcus aureus* in Fermented Sausage

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ABSTRACT. This study was carried out to determine the effect of salt application at different rates (1, 1,5, 2, and 2,5%) on the ability of *S. aureus* to produce enterotoxin in fermented sausage, one of the most produced meat products in Turkey. Five different reference strains of *S. aureus* known to have the ability to produce A-, B-, C-, D- and E-type toxin were inoculated into the experimentally produced sausage dough at 10^6 cfu g^{-1}. Chemical analysis of sausage samples were performed. The identification of *S. aureus* was confirmed by cultural and molecular methods. ELISA was used to determine the ability to produce enterotoxin, and classical PCR and multiplex PCR were used to detect enterotoxin genes. According to the analysis results, the moisture, a_w and pH values of the sausage samples were 38,80-61,48%; 0,883-0,901; 5,41-6,33, respectively. In production groups, *S. aureus* count was detected between 4,59-6,52 log cfu g^{-1} on day 0; 6,41-8,92 log cfu g^{-1} on day 7; 7,23-8,91 log cfu g^{-1} on day 14 and 6,13-8,82 log cfu g^{-1} on day 21. It was determined that bacterial counts reached the highest levels on the 7th day (after fermentation) in all groups ($p < 0,05$). Despite the logarithmic increase observed in *S. aureus* numbers in sausage samples, no toxin production could be detected. This situation can be explained by the lack of enviromental conditions required for enterotoxin production, the presence of competitive microflora, the fact of although the strains are enterotoxigenic, there is no expression-dependent production in the food matrix, and the fermentation conditions in sausage do not provide the neccessary environment for enterotoxin production. As a result, the high bacterial counts detected in sausage samples pose a potential risk to public health. Although salt levels have a limited effect on enterotoxin production, other factors, especially the hurdle factors in sausage, help prevent enterotoxin production. This makes fermented sausage an important food in terms of food safety. In addition to ensuring adequate hygiene for traditional Turkish type fermented sausage production, attention should be paid to incorporating practices such as Hazard Analysis Critical Control Point (HACCP) and Good Manufacturing Practices (GMPs) at every stage of the production process, i.e. from farm to table.

Keywords: Enterotoxin; fermented sausage; salt; *Staphylococcus aureus*.

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Introduction

Fermented foods are functional foods in which various enzymatic changes occur as a result of fermentation of foods through controlled processes using starter cultures and final products that are beneficial to health are formed. Fermentation is a food preservation and production method that has been practiced since ancient times (Akdeniz Oktay & Özbaş, 2020). In this respect, fermented meat products have historically been associated with the meat preservation process (Fraqueza & Patarata, 2020). When it comes to fermented meat products in Turkey, the first thing that comes to mind is the traditional Turkish type of fermented sausage (Konuray & Erginkaya, 2020). Fermented sausage production technology includes the stages of mincing meat and animal fat, adding salt, sugar, garlic, various spices and additives, filling the dough into natural or artificial casings, fermenting under certain temperature and relative humidity conditions and maturing/drying (Sucu & Turp, 2018; Armutcu et al., 2020; Benli et al., 2024). Many foods, especially meat and meat products, can spoil in a short time, especially due to microbial growth. Different preservation methods and the use of additives have been preferred to prevent this situation (De Carvalho et al., 2017). The leading ones among these are the methods using salt (Webster et al., 2011). Changing lifestyles, dietary habits and developments in food processing methods are the main factors in the increase in salt consumption. Considering the relationship between salt consumption and hypertension, dietary salt intake emerges as an important public health problem. In this sense, the importance of limiting salt intake is increasing day by day.

Among the bacteria that are tolerant to salt, *Staphylococcus aureus (S. aureus)* is the most important pathogen in sausages and other fermented meat products that causes intoxication and threatens public health (Kang & Fung, 2000; Attien et al., 2014; Nacer et al., 2024). Among the various foods investigated, meat and meat-based products are considered as the common main reservoir for *S. aureus* (Wu et al., 2018). Contamination of meat products is due to poor hygiene practices during processing and storage (Pérez-Boto et al., 2023). Adequate heat treatment during food processing can generally eliminate all vegetative *S. aureus* strains. However, SEs cannot be destroyed by heat treatment because they are thermostable and resistant to gastrointestinal proteases (Alves et al., 2018). Staphylococcal enterotoxins (SEs) are exoproteins that are produced by *S. aureus* in foods during the exponential phase of growth or during the transition from the exponential phase to the stationary phase and are responsible for SFP, which causes symptoms of acute gastroenteritis when consumed by humans (Argudín et al., 2010; Argaw & Addis, 2015).

SFP is a common foodborne illness globally caused by staphylococcal enterotoxins (SEs) (Gebremedhin et al., 2022). It is stated that toxins are low molecular weight (approximately 27-31 kDa), proteins consisting only of amino acids and are generally produced by CoPS species (Elshebrawy et al., 2025). Toxins are affected by environmental characteristics such as temperature, salt concentration, pH value (Landgraf & Destro, 2013). For SE production, the contamination level of coagulase positive *S. aureus* strain should be higher than 10^5 cfu g mL^{-1} (Bulajic et al., 2017; Şanlıbaba, 2022). *S. aureus* pathogenicity is mediated by a variety of virulence factors, which include coagulase, thermonuclease, exfoliative toxins A & B, enterotoxins, hemolysins (α, β, δ, and γ), toxic shock syndrome toxin-1 (TSST-1), and Panton-Valentine leukocidin (PVL), which help the bacterium in colonization, adherence, tissue invasion, and host cell lysis (Mahros et al., 2021; Şanlıbaba, 2022). The thermostable nuclease gene (*nuc*) has been employed as a marker for detecting *S. aureus* isolates in food and clinical samples (Elshebrawy et al., 2025). *S. aureus* often produces two groups of enterotoxin, namely new non-classical enterotoxins and classical enterotoxins (*sea, seb, sec*, *sed*, and *see*), which are the most prevalent and encoded by enterotoxin genes indicated as *sea* to *see* (Wang et al., 2017). Consumption of classical enterotoxins can cause many food poisoning outbreak cases, which are often self-limiting conditions that typically resolve within 24 h and are characterized by nausea, vomiting, fever, and diarrhea; however, these symptoms may worsen, particularly in the elderly and children (Tarekgne et al., 2016).

There are only a few studies reporting the prevalence of *S. aureus* in raw red meat in Turkey (Can et al., 2017; Koluman et al., 2011; Keyvan & Özdemir, 2016). However, there is no information about the prevalence of SEs in fermented sausages, a traditional meat product, according to different salt ratios. Therefore, it is important to investigate this issue further. The main objectives of this study were i) to perform the production of fermented sausages with salt reduction, ii) to determine the ability of *S. aureus* to produce enterotoxin, iii) to determine the presence of enterotoxin-encoding genes, and iv) to evaluate the food safety of Turkish-type fermented sausage, a traditional product.

Materials and methods

In this study, the meat and fat used as raw materials in the experimentally produced sausages were obtained from a butcher operating in Konya; spices and sausage casings were obtained from Prof. Dr. O. Cenap TEKİNŞEN Meat and Dairy Products Research-Development-Application Unit. In sausage production technology, the first meat used as raw material was analyzed for *S. aureus*. It was confirmed that no growth occurred in the meat samples cultivated in BPA medium. In order to determine the ability to form toxins, sausage production was carried out using different salt ratios. The experimental sausage production formulation is shown in Table 1. (Tekinşen & Doğruer 2000; Kaban et al., 2022).

Table 1. Sausage Production Formulation.

Meat+Fat Mixture	88+12 kg
Sodium nitrite	%0.015
Ascorbic acid	%0.5
Sugar	%0.5
Garlic	%1.5
Cumin	%1.5
Red pepper	%0.5
Pimento	%1
Black pepper	%0.5
Allspice	%0.6

Production was carried out in triplicate. After the sausage dough was prepared, it was placed in vacuum bags and grouped according to salt ratios and *S. aureus* enterotoxigenic strains. Six groups of sausage samples were prepared, including one control (K) and five experimental groups (SEA, SEB, SEC, SED, SEE). Each group was divided into subgroups containing four different salt ratios (1%, 1.5%, 2%, 2.5%) and subjected to analysis on days 0, 7, 14 and 21. For each group, four samples were analyzed on four different days (4*4) 16; for six groups, a total of (16*6) 96 samples were analyzed both chemically and microbiologically.

Inoculation of samples

Inoculation of reference strains into samples was performed by modifying the procedure of Yamazaki et al. (2008). 250 g of prepared sausage samples were taken into sterile stomacher bags. Fresh exponential growth phase was attempted to be captured by transferring the strains from 18-24 h cultures at 37°C to TSB. Bacterial count values of each strain in µL were adjusted according to McFarland values. Approximately 10^6 cfu g^{-1} bacteria in the exponential growth phase were transferred to sterile Eppendorf tubes from the culture suspension. Each group was inoculated in a biosafety cabinet so that 991 µL MRD+9 µL would be the reference strain. After inoculation, sausages were filled into casings. The processes used in sausage production are shown in Table 2. In the following days, the samples were stored in the cabinet at +4°C.

Table 2. Processes used in sausage production (Tekinşen & Doğruer, 2000; Yalçın & Ertürkmen, 2024).

	Temperature	Moisture	Air Circulation
Day 0	22 - / + 2°C	%95	0,5-0,8 m sn^{-1}
1st and 3rd day	22 - / + 2°C	%90	0,5-0,8 m sn^{-1}
4th day	22 - / + 2°C	%83	0,5-0,8 m sn^{-1}
5th and 6th days	18 - / + 2°C	%80	0,1-0,2 m sn^{-1}

Chemical analysis

To determine the moisture content of the samples, sausage samples weighed in nickel containers were dried in an oven and then allowed to cool in a desiccator. After cooling, the moisture content (%) were calculated (Mauer, 2024). The a_w value of the samples was determined using the a_w device (Novacina, Labswift-a_w) (Mauer, 2024). pH was measured using a digital pH meter (WTW, inoLab 720), which was calibrated with standard buffer solutions (pH 4.0 and pH 7.0) prior to use.

Microbiological analysis

Isolation and identification of *S. aureus*

A 10 g sample was taken from the sausage dough produced under aseptic conditions, 90 mL of MRD solution was added and then homogenized for 2 min using a stomacher. 1 mL of this homogenate was taken and serial dilutions from 10^{-1} to 10^{-8} were prepared and inoculations were performed on BPA using the pour plate method. It was incubated at 37°C for 24-48 hours (Normanno et al., 2005). Colonies with a diameter of 2-3 mm on the agar surface and gray-black zones around them were evaluated as suspicious for *S. aureus*. After incubation, five typical (grey-black, shiny, convex, 1-3 mm in diameter, with a clear zone around them) and/or atypical colonies were selected, and tube coagulase tests were performed using EDTA coagulase plasma (Merck, 1.13306.0001) (Bennett & Lancette, 1998). Following this, colonies with positive coagulase test were analyzed in terms of Gram stain, catalase test, DNase activity, mannitol fermentation test, and colonies with positive test results were evaluated as *S. aureus* (Kateete et al., 2010; Amini et al., 2011).

DNA isolation

DNA isolation was performed by modifying the protocol of Murphy et al. (2002). 18 mL of ethylenediaminetetraacetic acid (EDTA; VWR Chemicals, Leuven, Belgium, 20302.293, 0.5 M, pH 8) and 12 mL of Tris-EDTA (TE; Sigma-Aldrich, 77–86–1, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to 2 mL of sausage sample filtrate and shaken for 15 min at room temperature. Then, it was centrifuged at 4,000 rpm for 20 min. The pellet was resuspended in 1 mL of lysis buffer (100 mM Tris-HCl pH 8, 200 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 5 mM EDTA pH 8) and 15 µL of Proteinase K (Zymo, D3001–2; 20 mg mL^{-1}, Zymo Research, Irvine, CA, USA) and incubated overnight at 55°C. After

incubation, 1 mL of phenol-chloroform-isoamyl alcohol (25:24:1, SigmaAldrich, A2279, St Louis, MO, USA) was added to the solution and centrifuged at 12,500 rpm for 10 min. After phase separation, 150 µL sodium acetate (3M, pH 5.2) and 400 µL ethanol were added to the upper phase, and DNA was precipitated by centrifugation. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 40 µL TE. Isolated DNA was stored at -20°C until analyzed.

Detection of enterotoxin genes in *S. aureus* isolates

Primer sequences and amplification conditions used in the study are given in Table 3.

Table 3. Primer sequences and amplification conditions used in the study.

Gene	Primer sequence (5'- 3')	Amplicon size (bp)	Amplification conditions	References
sea	F: TTGGAAACGGTTAAAACGAA R: GAACCTTCCCATCAAAAACA	120	94°C 2 min, 94°C 30 s, 52°C 30 s, 72°C 45 s (40 cycles) 72°C 7 min	Johnson et al. (1991)
seb	F: TCGCATCAAACTGACAAACG R: GCAGGTACTCTATAAGTGCC	478	94°C 2 min, 94°C 30 s, 54°C 30 s, 72°C 45 s (40 cycles) 72°C 7 min	Johnson et al. (1991)
sec	F: GACATAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCC	257	94°C 2 min, 94°C 30 s, 48°C 30 s, 72°C 45 s (40 cycles) 72°C 7 min	Johnson et al. (1991)
sed	F: CTAGTTTGGTAATATCTCCT R: TAATGCTATATCTTATAGGG	317		
see	F: TAGATAAAGTTAAAACAAGC R: TAACTTACCGTGGACCCTTC	170		
seg	F: AAGTAGACATTTTTGGCGTTCC R: AGAACCATCAAACTCGTATAGC	287	94°C 2 min, 94°C 30 s, 55°C 30 s, 72°C 60 s (30 cycles) 72°C 7 min	Omoe et al. (2002)
seh	F: GTCTATATGGAGGTACAACACT R: GACCTTTACTTATTTCGCTGTC	213		
sei	F: GGTGATATTGGTGTAGGTAAC R: ATCCATATTCTTTGCCTTTACCAG	454		

PCR protocol and amplification used for detection of enterotoxin genes

PCR was performed by preparing a mixture in 25 µL volume. The reaction mixture contained 13.75 µL nuclease-free water, 5 µL MyTaq Reaction Buffer (Bioline BIO-21126), 1 µL of each primer (forward and reverse, 10 mM) specific for each enterotoxin gene, 1.5 U Taq DNA polymerase, and 5 µL of DNA template. Amplification products were electrophoresed on a 1.5% agarose gel at 100 V for 30 min. Specific DNA bands were identified by comparison with the positive control and DNA marker and visualized under a UV transilluminator.

Detection of Toxin Production of *S. aureus* Isolates Containing Enterotoxin Gene by ELISA Technique

In this study, R-Biopharm RIDASCREEN SET A, B, C, D, E kit was used to detect five classical enterotoxins. Bacterial cultures were first incubated in BHI Broth at 37°C for 24h, and 1 mL of culture was transferred to Eppendorf tubes. The tubes were centrifuged at 3,500 rpm for 7 min at 15°C. The supernatant was collected using a sterile syringe, passed through 0.22 µm diameter filters and transferred to new Eppendorf tubes. Then, 100 µL of each sample was added to the ELISA wells (except well H), and the subsequent steps were performed according to the kit instructions. Absorbance was measured with an ELISA reader at 450/620 nm. The cut-off value was determined by adding 0.15 to the arithmetic mean of the Optical Density (OD) values of the negative controls for each sample. For result validation, the positive control had to be ⩾ 1.0 and the negative control ⩽ 0.2. Assays that did not meet these criteria were considered invalid and repeated. In valid tests, if the absorbance of a sample at 450 nm was below the cut-off, it was considered negative, whereas values equal to or above the cut-off were considered positive.

Reference strains

In this study, SEA *S. aureus* ATCC 29213, SEB *S. aureus* NCTC 10654 FDA 243, SEC *S. aureus* NCTC 10655 137, SED *S. aureus* NCTC 10656 494, SEE *S. aureus* FRI 918 strains with known enterotoxigenic properties were used as reference strains.

Statistical analysis

In the study, descriptive statistics were performed for the measured sausage samples and shown as Arithmetic Mean ± Standard Error. Before significance testing, data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. *S. aureus* counts in sausage samples on days 0, 7, 14, and 21 were expressed as log cfu g^{-1}. Since the control groups did not have bacteria count results and the chemical analysis results were similar to the other samples, they were not included in the statistical analysis. In the examination of the differences between strains and rates of the changes in the criteria of bacterial count variables over time, three-way ANOVA was applied using the General Linear Model (GLM). The model included the interaction terms of time (within subjects), rate (between subjects) and strain*rate, time*strain, time*rate, time*strain*rate. Significant interactions were further analyzed using simple effects with Bonferroni correction. Statistical analyses were performed with SPSS version 29.0, and a significance level of $p < 0.05$ was applied for all tests.

Results and discussion

The moisture analysis results of the sausage samples produced on the 0th, 7th, 14th and 21st days are shown in Figure 1., a_w values in Figure 2., pH value in Figure 3. and Table 4. and statistical results of *S. aureus* counts in Table 5.

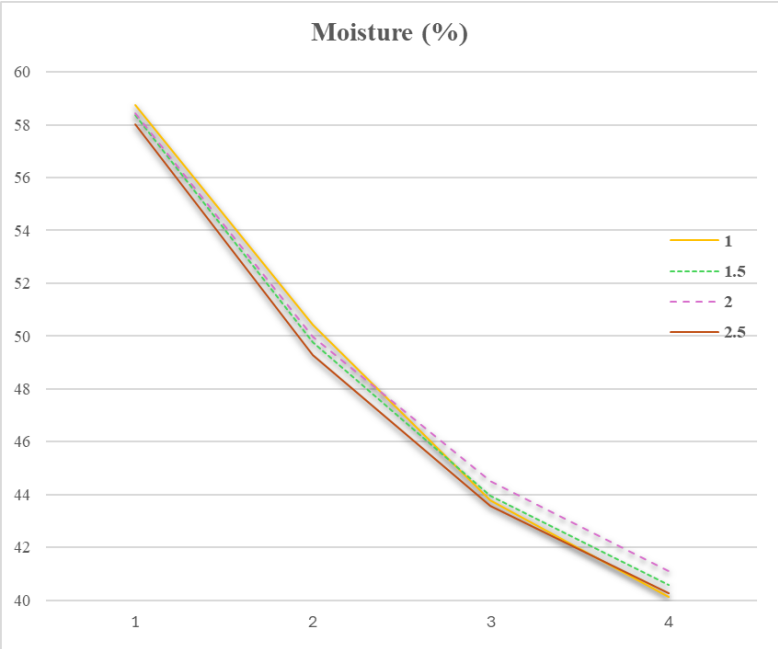

Figure 1. Moisture content changes of sausage samples according to days.

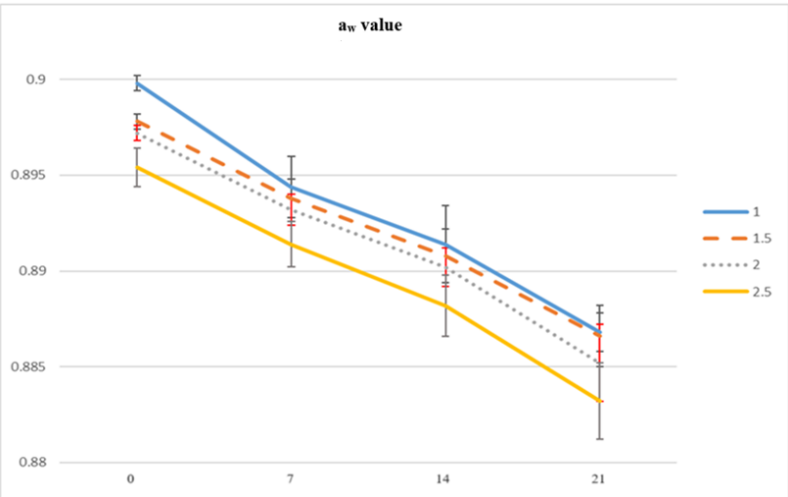

Figure 2. Changes in a_w of sausage samples according to days.

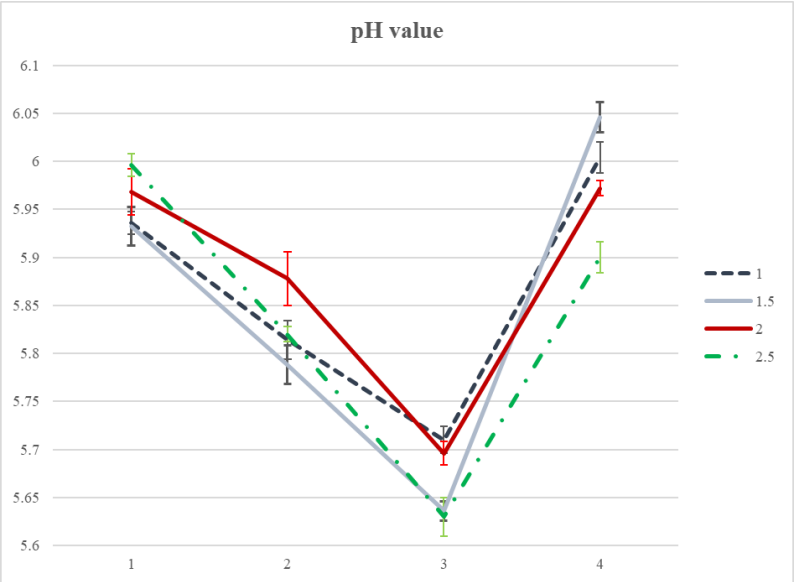

Figure 3. pH changes of sausage samples according to days.

Moisture contents of sausage samples were determined as 55.19-61.48% on day 0; 47.26-53.43% on day 7; 42.11-46.13% on day 14 and 38.80-42.35% on day 21, respectively.

The a_w values of the sausage samples were determined as 0.901-0.894 on day 0; 0.896-0.890 on day 7; 0.893-0.887 on day 14 and 0.890-0.881 on day 21, respectively.

Table 4. Changes in pH values of sausage samples.

Group	Salt Ratio (%)	Sampling Time			
		0th day	7th day	14th day	21st day
SEA	1	6,04 ± 0,00 $^{ab, \beta, B}$	5,96 ± 0,03 $^{b, \gamma, A}$	5,91 ± 0,01 $^{b, \delta, A}$	6,18 ± 0,01 $^{c, \gamma, C}$
	1,5	5,98 ± 0,01 $^{a, \beta, C}$	5,85 ± 0,03 $^{a, \beta, B}$	5,77 ± 0,00 $^{a, \gamma, A}$	6,09 ± 0,02 $^{b, \gamma, D}$
	2	6,05 ± 0,02 $^{b, \beta, C}$	5,97 ± 0,04 $^{b, \beta, B}$	5,85 ± 0,02 $^{b, \beta, A}$	6,11 ± 0,01 $^{b, \gamma, C}$
	2,5	6,06 ± 0,01 $^{b, \beta, C}$	5,96 ± 0,01 $^{b, \beta, B}$	5,73 ± 0,07 $^{a, \gamma, A}$	5,93 ± 0,01 $^{a, \beta, B}$
SEB	1	5,74 ± 0,04 $^{a, \alpha, C}$	5,61 ± 0,04 $^{a, \alpha, B}$	5,50 ± 0,02 $^{a, \alpha, A}$	5,54 ± 0,01 $^{a, \alpha, AB}$
	1,5	5,86 ± 0,02 $^{b, \alpha, B}$	5,80 ± 0,01 $^{b, \alpha\beta, B}$	5,67 ± 0,02 $^{c, \beta\gamma, A}$	6,14 ± 0,02 $^{c, \gamma\delta, C}$
	2	5,97 ± 0,03 $^{c, \beta, B}$	5,91 ± 0,01 $^{c, \alpha\beta, AB}$	5,83 ± 0,01 $^{d, \beta, A}$	5,99 ± 0,01 $^{b, \beta, B}$
	2,5	5,95 ± 0,01 $^{c, \alpha, C}$	5,82 ± 0,00 $^{bc, \alpha\beta, B}$	5,59 ± 0,01 $^{b, \beta, A}$	5,94 ± 0,04 $^{b, \beta, C}$
SEC	1	6,02 ± 0,01 $^{a, \beta, C}$	5,93 ± 0,01 $^{b, \gamma, B}$	5,74 ± 0,01 $^{b, \gamma, A}$	6,33 ± 0,03 $^{d, \delta, D}$
	1,5	5,97 ± 0,01 $^{a, \beta, C}$	5,75 ± 0,02 $^{a, \alpha, B}$	5,59 ± 0,01 $^{a, \beta, A}$	5,98 ± 0,02 $^{a, \beta, C}$
	2	6,01 ± 0,01 $^{a, \beta, C}$	5,82 ± 0,01 $^{ab, \alpha, B}$	5,63 ± 0,01 $^{a, \alpha, A}$	6,20 ± 0,01 $^{c, \delta, D}$
	2,5	6,10 ± 0,01 $^{b, \beta, C}$	5,73 ± 0,01 $^{a, \alpha, B}$	5,60 ± 0,00 $^{a, \beta, A}$	6,05 ± 0,01 $^{b, \gamma, C}$
SED	1	6,07 ± 0,01 $^{b, \beta, B}$	5,83 ± 0,01 $^{a, \beta, A}$	5,77 ± 0,02 $^{b, \gamma, A}$	6,13 ± 0,01 $^{b, \gamma, B}$
	1,5	6,00 ± 0,05 $^{ab, \beta, C}$	5,88 ± 0,03 $^{a, \beta, B}$	5,74 ± 0,01 $^{b, \gamma, A}$	6,16 ± 0,01 $^{b, \delta, D}$
	2	5,95 ± 0,03 $^{a, \beta, C}$	5,86 ± 0,06 $^{a, \alpha, B}$	5,59 ± 0,01 $^{a, \alpha, A}$	5,93 ± 0,00 $^{a, \beta, BC}$
	2,5	5,95 ± 0,01 $^{a, \alpha, B}$	5,90 ± 0,01 $^{a, \beta, B}$	5,79 ± 0,01 $^{b, \gamma, A}$	5,87 ± 0,01 $^{a, \beta, B}$
SEE	1	5,81 ± 0,00 $^{a, \alpha, C}$	5,74 ± 0,01 $^{a, \beta, B}$	5,63 ± 0,01 $^{b, \beta, A}$	5,84 ± 0,02 $^{c, \beta, C}$
	1,5	5,85 ± 0,01 $^{ab, \alpha, C}$	5,66 ± 0,01 $^{a, \alpha, B}$	5,41 ± 0,01 $^{a, \alpha, A}$	5,86 ± 0,01 $^{c, \alpha, C}$
	2	5,86 ± 0,03 $^{ab, \alpha, B}$	5,83 ± 0,02 $^{b, \alpha, B}$	5,58 ± 0,01 $^{b, \alpha, A}$	5,63 ± 0,01 $^{a, \alpha, A}$
	2,5	5,92 ± 0,02 $^{b, \alpha, C}$	5,69 ± 0,01 $^{a, \alpha, B}$	5,44 ± 0,01 $^{a, \alpha, A}$	5,71 ± 0,01 $^{b, \alpha, B}$
		Time, Strain, Ratio < 0,001 Strain*Ratio < 0,001 Time*Strain < 0,001 Time*Ratio < 0,001 Time*Strain*Ration < 0,001			

ABC: Same line, abc: Same column, αβγ: Different letters in the same column for each toxin strain when the salt ratio is the same are statistically significant ($p < 0,05$).

When the pH values of the samples were considered, the differences between days were statistically significant. In addition, the effects of strain, ratio, strain*ratio, time*strain, time*ratio and time*strain*ratio interactions were also significant ($p < 0.05$; Table 2.). The pH values of the sausage samples were found to be between 5.74-6.10 on day 0; 5.61-5.96 on day 7; 5.41-5.91 on day 14 and 5.54-6.33 on day 21, respectively.

Table 5. Change in the number of *S. aureus* in inoculated sausage samples (log cfu g^{-1}).

Group	Salt Ratio (%)	Sampling Time			
		0th day	7th day	14th day	21st day
SEA	1	4,63 ± 0,27 a, x, A	7,76 ± 0,57 a, x, B	7,53 ± 0,20 a, x, B	7,49 ± 0,40 a, xy, B
	1,5	5,36 ± 0,35 a, x, A	8,81 ± 0,04 b, y, C	8,19 ± 0,01 b, y, BC	7,73 ± 0,33 a, xy, B
	2	4,59 ± 0,14 a, x, A	7,34 ± 0,06 a, x, B	7,52 ± 0,12 a, x, B	7,24 ± 0,05 a, xy, B
	2,5	5,17 ± 0,37 a, x, A	7,21 ± 0,02 a, x, B	7,94 ± 0,01 ab, x, C	7,09 ± 0,05 a, x, BC
SEB	1	6,32 ± 0,03 a, y, A	8,73 ± 0,02 a, y, B	7,36 ± 0,22 a, x, AB	7,01 ± 0,36 a, xy, AB
	1,5	6,01 ± 0,08 a, xy, A	7,94 ± 0,36 a, y, B	7,86 ± 0,03 a, xy, B	6,82 ± 0,11 a, x, A
	2	6,13 ± 0,01 a, y, A	8,60 ± 0,01 a, y, C	7,89 ± 0,01 a, x, B	8,82 ± 0,60 b, z, C
	2,5	5,69 ± 0,11 a, x, A	8,71 ± 0,02 a, y, C	7,91 ± 0,09 a, x, B	7,35 ± 0,33 a, x, B
SEC	1	6,50 ± 0,14 a, y, A	8,79 ± 0,01 b, y, B	8,57 ± 0,05 a, y, B	8,50 ± 0,19 a, y, B
	1,5	6,50 ± 0,44 a, y, A	8,85 ± 0,32 b, y, B	8,25 ± 0,15 a, y, B	8,24 ± 0,09 a, y, B
	2	6,23 ± 0,17 a, y, A	8,05 ± 0,36 b, xy, B	8,72 ± 0,32 a, y, C	7,83 ± 0,24 a, yz, B
	2,5	5,48 ± 0,10 a, x, A	7,16 ± 0,03 a, x, B	8,32 ± 0,12 a, x, C	7,65 ± 0,09 a, x, BC
SED	1	6,50 ± 0,05 a, y, A	8,78 ± 0,02 b, y, B	7,55 ± 0,13 a, x, AB	6,95 ± 0,18 a, x, A
	1,5	6,41 ± 0,15 a, y, A	6,41 ± 0,21 a, x, A	8,33 ± 0,14 ab, y, B	7,59 ± 0,22 a, xy, B
	2	6,52 ± 0,27 a, y, A	8,92 ± 0,00 b, y, C	8,91 ± 0,01 b, y, C	7,57 ± 0,33 a, y, B
	2,5	5,71 ± 0,06 a, x, A	6,86 ± 0,29 a, x, B	8,09 ± 0,32 a, x, C	7,48 ± 0,16 a, x, BC
SEE	1	5,77 ± 0,16 a, y, A	7,31 ± 0,24 a, x, B	7,23 ± 0,04 a, x, B	8,04 ± 0,54 b, xy, B
	1,5	6,46 ± 0,53 a, y, A	8,61 ± 0,01 b, y, B	7,40 ± 0,38 ab, x, AB	7,33 ± 0,17 b, xy, A
	2	6,28 ± 0,03 a, y, A	6,86 ± 0,15 a, x, AB	7,33 ± 0,17 a, x, B	6,13 ± 0,09 a, x, A
	2,5	6,10 ± 0,01 a, x, A	7,15 ± 0,08 a, x, B	8,00 ± 0,05 b, x, C	7,83 ± 0,31 b, x, BC
Time, Strain, Ratio < 0,001 Strain*Ratio < 0,001 Time*Strain < 0,001 Time*Ratio < 0,001 Time*Strain*Ratio < 0,001					

ABC: Same line, abc: Same column, αβγ: Different letters in the same column for each toxin strain when the salt ratio is the same are statistically significant (p < 0,05).

Considering the change in *S. aureus* counts, the differences between days were statistically significant, while strain, ratio, strain*ratio, time*strain, time*ratio and time*strain*ratio interactions were found to be significant ($p < 0.05$). *S. aureus* counts in sausage samples inoculated with SEA, SEB, SEC, SED and SEE strains were detected between 4.59-8.81 log cfu g^{-1}; 5.69-8.82 log cfu g^{-1}; 5.48-8.72 log cfu g^{-1}; 5.71-8.92 log cfu g^{-1} and 5.77-8.61 log cfu g^{-1}, respectively.

The presence of enterotoxin genes in sausage inoculated with different *S. aureus* strains was detected by PCR. Multiplex PCR images of *S. aureus seg* and *sei* enterotoxin genes are shown in Figure 4. The *seh* gene was not detected in any of the samples. No SE production was detected by ELISA in sausage samples inoculated with classical enterotoxin-producing *S. aureus* strains. ELISA results of reference strains and sausage isolates are shown in Table 6.

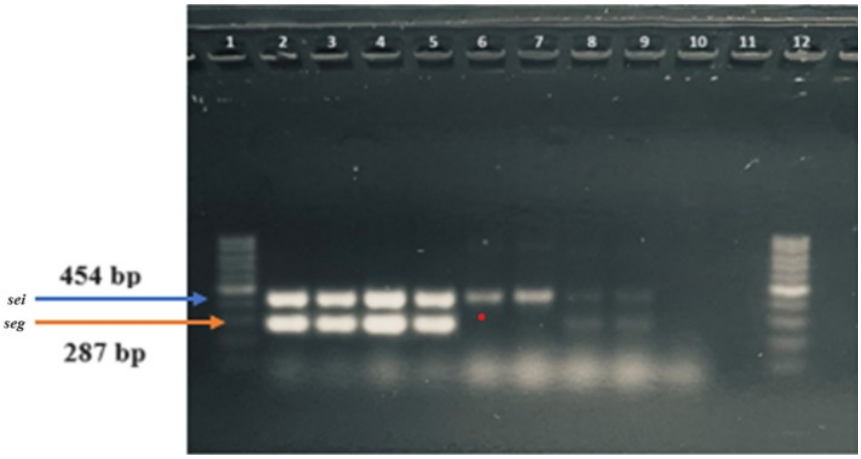

Figure 4. Agarose gel image of *seg* and *sei* genes (1, 12: 100 bp Ladder, 2: A1; 3: A1,5; 4: A2; 5: A2,5; 6: C1; 7: C1,5; 8: E1; 9: E1,5; 10, 11: Negative control).

Table 6. Values of classical enterotoxins determined at 450 nm wavelength in spectrophotometer device.

Toxin Type	Well	Reference Strains					Sausage Isolates						
		1	2	3	4	5	6	7	8	9	10	11	12
SEA	A	3,390	0,109	0,064	0,043	0,085	0,117	0,046	0,045	0,054	0,060	0,050	0,041
SEB	B	0,055	2,059	0,097	0,040	0,115	0,073	0,066	0,033	0,040	0,045	0,043	0,039
SEC	C	0,069	0,078	2,498	0,081	0,096	0,072	0,049	0,039	0,037	0,039	0,040	0,045
SED	D	0,036	0,196	0,156	2,956	0,211	0,094	0,129	0,047	0,063	0,055	0,058	0,048
SEE	E	0,079	0,096	0,058	0,074	2,651	0,074	0,187	0,043	0,044	0,045	0,041	0,043
NK	F	0,049	0,065	0,061	0,051	0,095	0,084	0,043	0,039	0,044	0,042	0,039	0,046
NK	G	0,051	0,065	0,061	0,055	0,142	0,084	0,045	0,037	0,042	0,038	0,038	0,040
PK	H	2,241	2,007	2,126	2,265	2,381	1,783	1,852	1,840	1,446	1,465	1,648	1,481
Cut-off Value		0,200	0,215	0,211	0,203	0,268	0,234	0,194	0,188	0,193	0,190	0,188	0,193

1: SEA, 2: SEB, 3: SEC, 4: SED, 5: SEE are the optical density (OD) values of the isolate numbers of the strains. These are the OD values of some of the sausage samples inoculated with 6-12 strains.

Fermentation is the most critical stage of sausage production. Internal (salt and sugar amount, fat content, meat size, casing type, calibration, and microflora in the sausage) and external (temperature, moisture, and airflow) factors affect the fermentation process and final product directly (Kaban et al., 2022; Yilmaz Topcam et al., 2024). The findings of the chemical properties obtained in the study are consistent with the findings obtained in the field study of many researchers (Pehlivanoğlu et al., 2015; Keskin & Uçar, 2023; Yalçın & Ertürkmen, 2024). İnce et al. (2018), in their analysis of eight fermented sausages sold in Turkish supermarkets, reported a moisture content ranging from 35.63% to 51.48% with an average of 43.27%. The moisture content determined in this study was lower than the reported average on day 0 but became similar in the following days. Yilmaz Topcam et al. (2024) determined in their study that the moisture content of sausage dough varied between 60.95-62.28%. They reported that the decrease in moisture content in sausages was due to water loss during drying. a_w values of all sausage samples decreased on days 0, 7, 14, 21. It was reported that a_w of sausage values (min:0.880, max:0.950) for fermented sausage. pH and a_w are considered two major hurdle factors in sausages (Yilmaz-Oral & Sallan, 2023). Overall, when moisture, a_w, and pH were considered collectively, the findings of Hu et al. (2020) on dry fermented sausages with different salt levels (2.5%, 2.0%, 1.5%, and 1.0%) were consistent with the findings of the present study. Across the sampling days, the decreases in chemical properties were attributed to the drying process.

The pH of sausages affects structural and sensory qualities and also ensuring the product's microbiological safety (Yilmaz-Oral & Sallan, 2023). The environment in fermented sausages promotes the dominance of LAB and the production of lactic acid during fermentation. Therefore, the pH value of sausage sharply declines, in particular, in the first days of fermentation, as shown in Figure 3. The pH pattern we found complies with the study results obtained by Lorenzo et al. (2014), Kamiloğlu et al. (2019). In this study, it was observed that the pH values of the samples were similar to the findings obtained in the studies (Atasever et al., 1998; Keskin & Uçar, 2023). In general, when the groups are evaluated, it is observed that the lowest pH values were reached on the 14th day and the highest pH values were reached on the 21st day. The increase in pH observed in sausages was explained by Kumar et al. (2017) as the presence of salt in fermented meat products. In addition, Kaya & Kaban (2013) stated that the pH value increased due to the decarboxylation and deamination reactions of amino acids formed as a result of proteolysis or ammonia and amines formed by their advanced metabolism. In similar studies (González-Fernández et al., 2006; Campagnol et al., 2011), it was reported that the increase in pH values on the 21st day was due to ammonia production due to enzyme activity (proteolysis) during ripening, increase in buffer substances and decrease in electrolytes. Yilmaz Topcam et al. (2024) stated that insufficient LAB and high pH environments support the growth of *S. aureus*.

The *S. aureus* count results of the sausage samples examined, when all toxin groups were examined, the differences between the groups were found to be significant on the 7th and 14th days, while the difference in the samples inoculated only with the SEB strain on the 21st day was found to be remarkable (Table 5.). This situation can be explained by the fact that the development of *S. aureus* strains with enterotoxigenic properties continued at high levels on the 7th and 14th days. SEA is produced during the exponential phase of development. The highest concentration of SEB, SEC and SED is produced during the transition from the exponential to the stationary phase of development. This is expressed as a feature of their regulation by the *agr* system (Derzelle et al., 2009).

Although fermented meat products contain microbiologically safe microorganisms, they are environments where pathogenic microorganisms can grow (Yilmaz Topcam et al., 2024). *S. aureus* is a foodborne pathogen that can be found in fermented meat products. Retail product research has shown that *S. aureus* is frequently isolated from sausage. The first days of the fermentation process are significant for *S. aureus* development due to the pH, a_w, and the changing microbial flora (Kaban & Kaya, 2006). LAB are competitive flora for coagulase positive Staphylococci. Therefore, the presence of a high number of LAB in the environment is important for the inhibition of these microorganisms (Yilmaz Topcam et al., 2024). The high number of *S. aureus* in the raw material, the low numbers of LAB, the high-pH environment, and the high a_w are the main factors favoring the production of staphylococcal enterotoxins. Consequently, the processing environment, equipment, raw materials, and personnel can all serve as sources of *S. aureus* contamination in meat products (Fetsch & Johler, 2018).

Conventional detection methods for SEs include polymerase chain reaction, liquid chromatography-mass spectrometry and enzyme-linked immunosorbent assay (ELISA) (Xu et al., 2019; Wang et al., 2023; Li et al., 2024; Zhang et al., 2024a; Zhang et al., 2024b). These techniques are generally costly, time-intensive, and require sophisticated equipment and skilled operators, which limits their application for field and rapid screening of SEs (Sun et al., 2025). However, ELISA is a method used to determine classical enterotoxin types and, together with PCR-based methods, is sensitive to contamination during processing, thus avoiding false-positive results is important (Wang et al., 2025). Although a logarithmic increase in *S. aureus* counts was observed in sausage samples in the study, toxin production was not detected by ELISA. The ability of *S. aureus* to produce detectable amounts of enterotoxin in foods depends on whether the strain is enterotoxigenic or not and the presence of environmental conditions necessary for enterotoxin synthesis (Paulin et al., 2011). In addition, it is stated that it is also related to the lack of expression of enterotoxin genes (Gajewska et al., 2023). It is stated that *S. aureus* at a level of approximately 10^5-10^6 cfu g^{-1} is the minimum value required for SE to be detected in foods (Paulin et al., 2011). The production of some SEs (SEB, SEC, SED) is linked to Quorum Sensing through the *agr* system (Ortega et al., 2010). Therefore, it is stated that bacteria must be present in high concentrations for enterotoxin production to occur (Paulin et al., 2011). However, current studies on the subject (Johler et al., 2015; Schwendimann et al., 2021) show that the presence of enterotoxins can be detected even when this number is lower. Especially during the development of *S. aureus*, enterotoxins are produced in low amounts in the exponential phase, while their production may increase in the post-exponential and stationary phases. Bang et al. (2008) reported that enterotoxin was not detected in samples with 10^9-10^{10} cfu g^{-1} *S. aureus* counts in pork sausages produced by adding additional salt (1.76%; 2.24%; 3.64%) and sodium nitrite (without and with 154 ppm added). The researchers stated that a 2-3 log increase in *S. aureus* counts during the ripening process of the sausage is not a desirable situation in foods. This shows that environmental factors (e.g., drying and temperature) are effective in the formation of SE. The findings obtained in this study are consistent with the results of Bang et al. (2008). Achieving high *S. aureus* counts in the product does not necessarily mean that the microorganism can produce enterotoxin.

In their study, Paulin et al. (2011) reported that enterotoxin was produced in broth culture at a concentration of $3x10^6$ cfu mL^{-1}, while it did not form enterotoxin in commercial cheese at a concentration of 7-8x10^6 cfu mL^{-1}. It was stated that staphylococci can grow at high concentrations without producing enterotoxin. Cretenet et al. (2011) stated that high concentrations of enterotoxigenic *S. aureus* are required for enterotoxin formation, but other factors such as pH and a_w are also important in enterotoxin production.

Toxin production by bacteria may vary depending on the environment they are in (Márta et al., 2011). There are various studies on the use of food matrices instead of laboratory environments in determining the enterotoxin production ability of *S. aureus* strains (Derzelle et al., 2009; Even et al., 2009; Wallin-Carlquist et al., 2010; Cretenet et al., 2011; Valihrach et al., 2014). Processed meat products are widely contaminated by *S. aureus*. However, in the literature search on enterotoxin production and expression, sufficient scientific literature was not identified. However, there are studies on enterotoxin A and D production in processed pork (Wallin-Carlquist et al., 2010; Márta et al., 2011; Zeaki et al., 2014). Alibayov et al. (2015) investigated the effect of different meat types on enterotoxin formation and expression. In the study, a low level of correlation was found between the growth of *S. aureus* strains and enterotoxin expression and production in meat samples. Enterotoxin expression is generally associated with bacterial growth in both meat products and meat broth cultures (Zeaki et al., 2014). Alibayov et al. (2015) also stated that the sec expression level in BHI reached the highest level in the late exponential phase and then decreased, consistent with the findings of Wallin-Carlquist et al. (2010). Similar results were also found by Derzelle et al. (2009). In fact, the researchers suggested that the expression of sec and other toxins increased in the post-exponential growth phase under

planktonic growth conditions in laboratory conditions when the bacterial concentration reached 4×10^8 cfu mL^{-1} and 2×10^9 cfu mL^{-1}. Alibayov et al. (2015) stated that food matrices affect the development of *S. aureus* and enterotoxin production. In the research conducted with sausage samples, it was stated that rapid acidification prevented the development of *S. aureus* and toxin production (Kaban & Kaya, 2006).

In the study conducted by Attien et al. (2014) with samples obtained from retail sales points, although *S. aureus* was detected, enterotoxin production was not observed. Considering this situation, it was observed that the relevant findings were in accordance with the data of this study. It is thought that the existence of studies in which enterotoxin production was detected (Asgarpoor et al., 2018; Yalçın & Can, 2013) may be due to a wide variety of contamination sources, including personnel hygiene in the final product.

The development of *S. aureus* and enterotoxin production are affected by various factors such as the population density of *S. aureus*, the presence of competitive microflora, the use of LAB as a starter culture, the storage environment of the food, temperature, pH, salt, a_w values and the presence of oxygen. For *S. aureus*, the level of 10^6 cfu g^{-1} is important in terms of toxin production. In this study, since no starter culture was used to reduce acidity, fermentation was initiated by the naturally occurring LAB flora. It is thought that this situation may cause the fermentation to proceed more slowly and therefore the pH value may not decrease to the point where it prevents *S. aureus* development and toxin production. In addition, when the bacterial counts and gene expression are taken into account, it can be stated that enterotoxin detection may vary depending on the inhibitory factors in the sausage.

It was determined that all sausages produced by inoculating enterotoxigenic strains contained enterotoxin genes. Among these, in samples inoculated with SEA, SEC and SEE strains, it was determined that in addition to *sea, sec* and *see* genes, *sei* and *seg* genes were also present (Figure 4.). In the literature searches, no studies on toxin genes were found during the production period. In this sense, in some studies on the final product in the field (Gencay et al., 2010; Moustafa et al., 2016; Eid et al., 2018; EL-Maghraby et al., 2018; Mahfoozi et al. 2019; Savariraj et al., 2019; Sahin et al., 2020), gene profiles were tried to be determined. The high coexistence of *seg* and *sei* in *S. aureus* isolates suggests that most of these genes may be members of *egc* in positive isolates. The presence of SE genes in *S. aureus* isolates is required for these strains to cause food poisoning or other diseases. However, it is important to demonstrate that strains harboring these newly reported SE genes produce toxins at levels sufficient to cause disease (Omoe et al., 2002).

Conclusion

In the production of traditional Turkish-type fermented sausages, reducing salt levels may influence *S. aureus* growth. Salt inhibits microbial growth, and its reduction can diminish this protective effect, thereby increasing the risk of growth of *S. aureus*. *S. aureus* growth and enterotoxin production are also related to the pH value of the medium, temperature, a_w and other factors, and reducing the amount of salt may facilitate the growth of *S. aureus* by combining with the effects of these factors. Therefore, preventing *S. aureus* contamination in the sausage production process is of critical importance in terms of initial LAB count, pH reduction and sufficient fermentation. At the same time, the necessity of HACCP, GMP and GHP applications also comes to the fore. In the studies conducted, attention should be paid to the interaction of *S. aureus* with the food matrix and SE production, new and sensitive methods should be developed for SE detection.

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