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Effects of Autochthonous Starter Cultures on the Behavior of *Staphylococcus aureus* during the Production of a Semi-Dry Fermented Sausage

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HIGHLIGHTS

- A rapid pH drop during fermentation is of great importance for the control of *Staphylococcus aureus*.
- *Lactilactobacillus sakei* S15 was more effective on *S.aureus*.
- Enterotoxine could not be detected even when no starter culture was used.
- *L.sakei* S15 and *Lactiplantibacillus plantarum* S91 decreased the pH below 5,3 after 24 h of fermentation.

Abstract

Staphylococcus aureus can grow and produce enterotoxin during the production of fermented sausages, especially in the early stage of the fermentation. Furthermore, staphylococcal enterotoxins are heat-stable. Therefore, inhibiting the growth of this pathogen during production is of great importance for food safety. The study was carried out to determine the effects of autochthonous lactic acid bacteria (LAB) strains (*Lactiplantibacillus plantarum* S91, *Latilactobacillus sakei* S15 and *Pediococcus acidilactici* S147b) on the behavior of *S. aureus* in heat-treated sucuk (HTS) (raw fermented cooked and dried), a type of semi-dry fermented sausage. The HTS batters were inoculated with *S. aureus* ATCC 51740 (SEB) at 10^5 CFU/g level. In groups containing *L. sakei* S15, pH decreased faster in the first 24 h of fermentation (22 °C) than in other groups. After 48 h, pH dropped below 5.0 in all groups with autochthonous strains, while it was still above 5.5 in groups without autochthonous strains. Therefore, while the number of *S. aureus* increased during fermentation in the sausage group without autochthonous strains, there was no significant change in the number in the presence of autochthonous strains. The heat treatment (core temperature; 68 °C) caused significant reductions in *S. aureus*, LAB and *Micrococcus*/*Staphylococcus* (< 2 log CFU/g). At the end of drying (18 °C), the a_w value varied between 0.927 and 0.935. Staphylococcal enterotoxin was not detected in all groups. In conclusion, the rapid decrease in pH during the early stage of fermentation is an important hurdle effect in the controlling the growth of *S. aureus*.

Keywords: *Staphylococcus aureus*; Fermented sausage; Autochthonous strains; Heat treated sucuk; Staphylococcal enterotoxin

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1. Introduction

Foodborne pathogens have become an important social problem for consumers. They have also received much attention from consumers and food safety authorities around the world due to frequent outbreaks of microbial infections and intoxication (Gao et al. 2019). *Staphylococcus aureus* is an important pathogen for humans and animals. In addition to staphylococcal infections, *S. aureus* also causes staphylococcal intoxications through the enterotoxins (Titouche et al. 2020). The optimum growth temperature of *S. aureus* ranges from 30 to 37 °C, and it can grow in a wide range of temperature (7 to 48.5 °C) (Kadariya et al. 2014). In addition, this bacterium can show optimum growth between pH 6.0 and 7.0. *S. aureus* can tolerate salt and nitrite. However, this pathogen is a poor competitor under anaerobic conditions, at low pH and low temperatures (Holck et al. 2017; Lücke 1998). Another characteristic of this pathogen is sensitivity to heat treatment. The D value is approximately 1 min at 65 °C (Lücke and Troeger 2007). *S. aureus* produces a range of staphylococcal enterotoxins (SEs) which are a major cause of foodborne intoxications. SEs are resistant to freezing, drying, heat treatment, and low pH (Antoine Hennekinne et al. 2012; Loir et al. 2003). They are also resistant to proteolytic enzymes (Antoine Hennekinne et al. 2012). In addition, enterotoxins can form over a wide range of temperatures (10 - 48 °C), a_w (0.87 - 0.99), pH (4 - 10) and salt (1.7 - 17%). The optimum conditions for enterotoxin production in terms of temperature, a_w, pH and salt level are 37 °C, 0.98, 6-7 and 3.7 %, respectively (Bang et al. 2008).

The decreases in pH and a_w values fermentation and drying in fermented sausages are of great importance for product safety. These sausages play an important role in staphylococcal food poisoning (Ananou et al. 2005; Ferreira et al. 2006). The main reason for this is that the pH and a_w values of sausage batches are suitable for the growth of *S. aureus* (Hampikyan 2009; Lücke 1998; Sameshima et al. 1998). In the first days of fermentation, a critical stage for pathogenic microorganisms, *S. aureus* can grow well and produce enterotoxins in fermented sausages (Gonzalez-Fandos et al. 1999; Paramithiotis and Drosinos 2017; Rajkovic et al. 2017). All these points show that the growth of *S. aureus* during the early stages of fermentation is very important for microbiological stability in fermented sausages.

Heat-treated sucuk (HTS), a type of semi-dry fermented beef sausage, is made from beef or poultry. There are three consecutive processing stages in the production of this product: fermentation, heat treatment and drying. Considering that heat treatment has a significant contribution to product safety, the production of this product is increasing day by day (Kaban and Bayrak 2015). However, there is no information on the behavior of *S. aureus* in HTS. Therefore, the aim of this work was the evaluate the influence of autochthonous strains (*Lactiplantibacillus plantarum* S91, *Latilactobacillus sakei* S15 or *Pediococcus acidilactici* S147b) and their mixture on the behavior of *S. aureus* during processing of HTS, as well as determination of a_w, pH and numbers of *Micrococcus*/*Staphylococcus* and lactic acid bacteria (LAB). The study also examined the presence of enterotoxins in final products.

2. Materials and Methods

2.1. Material

Latilactobacillus sakei S15 (KR025387), *Lactiplantibacillus plantarum* S91 (KT327838), and *Pediococcus acidilactici* S147b (KT275957) strains (Kaya et al. 2017) were added to batters as starter cultures. *Staphylococcus aureus* ATCC 51,740 (SEB) strain was used for HTS batter contamination. Beef and beef fat were used in the production of the HTS batters.

2.2. Sausage Production

In the production of HTS, per kg meat and fat (80 % lean beef and 20 % meat fat) was used: 20 g salt, 4 g sucrose, 5 g black pepper, 2.5 g allspice, 10 g garlic, 9 g cumin and 7 g red pepper. Sodium nitrite (150 mg/kg) was used as curing agent. Three independent batches were prepared for each treatment: A: *S.aureus* and starter culture not inoculated, B: *S.aureus*, C: *S.aureus* / *L. plantarum*, D: *S. aureus* / *L. sakei*, E: *S. aureus* / *P. acidilactici*, F: *S. aureus* / *L. plantarum* / *L. sakei* / *P. acidilactici*. Thus, a total of eighteen batches were prepared. *S. aureus*

ATCC 51,740 was inoculated at 10^5 CFU/g, the autochthonous strains were added at 10^7 CFU/g into batches. The experiment was replicated three times. Thus, eighteen batches were prepared.

The HTS batters were prepareted in a small scale cutting machine (MADO, MTK 662, Germany). The batters prepared were filled into collagen casing (38 mm, Naturin Darm, Germany) using filling machine (MADO, MTK 591, Schwarzwald). After filling, the HTS samples were transferred to a climate chamber (Reich, Germany). After fermentation (relative humidity; 92 ± 2 %, temperature; 22 ± 1 °C, fermentation duratation; 24, 48 or 72 h.) the samples were subjected to the heat treatment (core temperature; 68 °C) in a cooking chamber (Mauting, Czech Republic). After this process, the samples were again transferred to the climate chamber for drying and dried (relative humidity; 84 ± 2 %, temperature; 18 ± 1 °C).

2.3. Sampling

The sampling was carried out during fermentation times (0, 24, 48 and 72 h). Sampling was also done after heat treatment and drying processes. The samples were subjected to the following microbiological and physicochemical analyzes.

2.4. pH and a_w Analysis

For the analyse pH value, 10 g of the sample was homogenized with 100 mL of distilled water for 1 min. The pH value was measured using a pH-meter (Mettler Toledo, Switzerland). Buffer solutions (pH 4 and 7) were used to calibrate the pH meter.

To determine the water activity (a_w) value the water activity device was used (Novasina, Model TH 500, Switzerland).

2.5. Microbiological Analysis

The spread plate method was used for the bacterial enumerations. Baird-Parker Agar (Merck, Germany) plates were used for the enumeration of *S. aureus*. After incubation (48 h, 37 °C), typical black colonies were subjected to the coagulase test. De Man Rogosa Sharpe Agar (Merck, Germany) and Mannitol Salt Phenol Red Agar (Merck, Germany) were used for the enumeration of the LAB and *Micrococcus*/*Staphylococcus*, respectively. MRS plates were incubated at 30 °C for 48 h under anaerobic jar (Anaerocult A, Merck, Germany). MSA plates were incubated at 30 °C for 48 h.

2.6. Enterotoxin Analysis

The presence of enterotoxins was determined using the VIDAS Staph enterotoxin II (Biomerieux, France) enzyme-linked fluorescent immunoassay (AOAC 2007).

2.7. Statistical Analysis

The autochthonous LAB and the processing stages were considered as factors. Three batches (replicates) of sausage were produced independently for each group. The autochthonous LAB was evaluated as fixed effect and three replications as random effect. All data were subjected to statistical analysis (Two-way ANOVA) using SPSS (Chicago, USA).

3. Results

3.1. pH and a_w

The changes in the pH of the HTS during the production are shown in Figure 1. The pH value was affected by the fermentation time (0, 24, 48 and 72h) ($p < 0.01$). No significant change in pH value was observed after 24 h of fermentation in A (*Staphylococcus aureus* and starter culture not inoculated) and B (*S. aureus* inoculated) groups. However, there was a slight decrease in pH value after 48 h of fermentation in these groups. As can be seen from Figure 1, a wide variation in pH value was observed. This result is thought to be due to the diversity of the microbiota of the raw material. To put it more clearly, a decrease in pH was observed depending on the spontaneous flora. A similar trend was observed after 72 h. The use autochthonous starter culture (mixed or mono) caused a significant decrease in pH value throughout fermentation. Lactic acid formation is the main reason for pH decrease. During the fermentation, the autochthonous LAB exhibited a

good growth produced lactic acid, which reduced the pH value of the sausages. The pH decrease in the first 24 h of fermentation is important for the inhibition of *S. aureus*. After 24 h of fermentation, the pH in group E with *Pediococcus acidilactici* S147b did not fall below 5.3. The result indicated that, *Latilactobacillus sakei* S15 and *Lactiplantibacillus plantarum* S91 showed better growth at applied fermentation temperature (22 °C) than *P. acidilactici* S147b. However, *L. sakei* S15 decreased pH more than *L. plantarum* S91. Lücke (1998) and Kaban et al. (2012) also reported that *L. sakei* is more effective than other LAB species in fermented sausages at initial fermentation temperature of 20 - 22 °C.

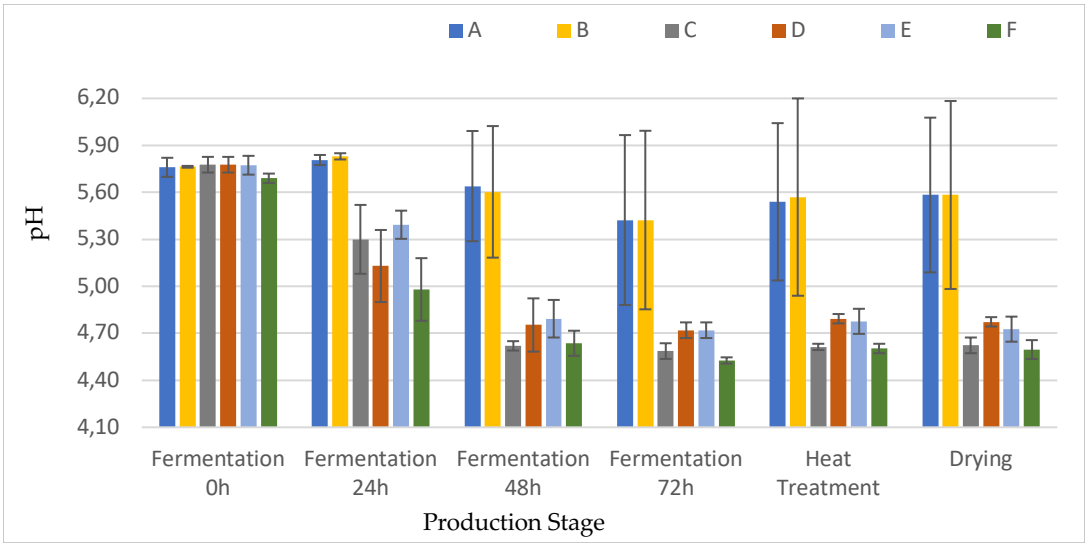

Figure 1. The changes in the pH value of HTS during the production (A: *S.aureus* and starter culture not inoculated, B: *S.aureus*, C: *S.aureus* / *L.plantarum*, D: *S.aureus* / *L.sakei*, E: *S.aureus* / *P.acidilactici*, F: *S.aureus* / *L.plantarum* / *L.sakei* / *P.acidilactici*).

The a_w values of HTS samples during the production are shown in Figure 2. The a_w value for all treatment at the beginning was found between 0.969 and 0.974. Fermentation time had a very significant effect on a_w value ($p < 0.01$). At the end of production (end product), a_w value was between 0.936 and 0.946. Among the hurdle effects such as nitrite, pH, redox potential and competing flora in fermented sausages, water activity plays an important role. Although *S. aureus* is a salt and nitrite resistant foodborne pathogen microorganism, it is very acid sensitive. pH along with a_w may play a more active role in inhibition of this pathogen (Kaya and Kaban 2019).

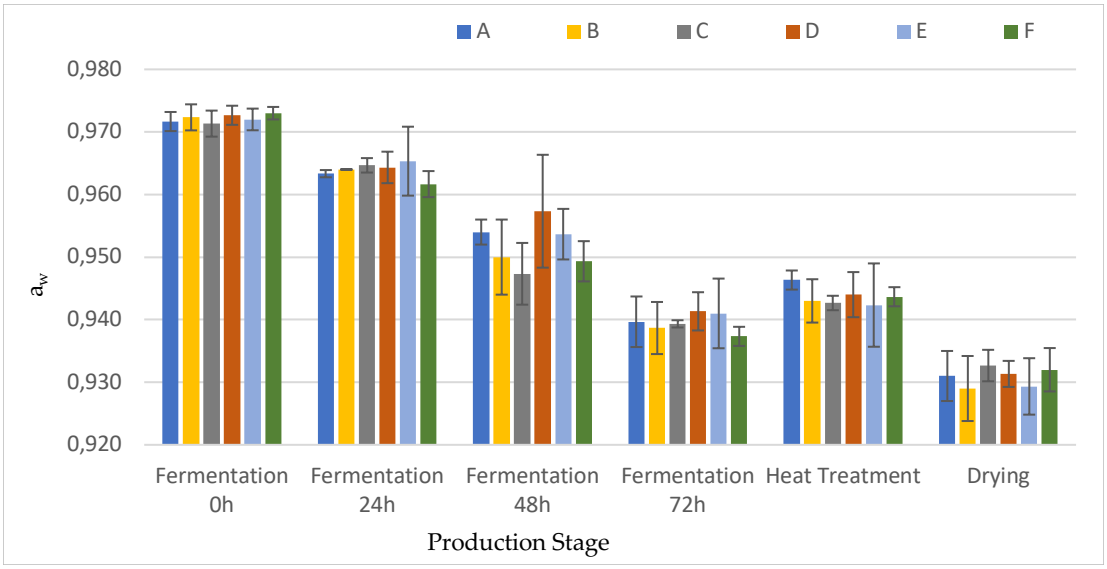

Figure 2. The changes in the a_w value of HTS during the production (A: *S.aureus* and starter culture not inoculated, B: *S.aureus*, C: *S.aureus* / *L.plantarum*, D: *S.aureus* / *L.sakei*, E: *S.aureus* / *P.acidilactici*, F: *S.aureus* / *L.plantarum* / *L.sakei* / *P.acidilactici*).

3.2. Staphylococcus aureus

The changes in the *S. aureus* of HTS during the production are shown in Figure 3. In the group A, the number of *S. aureus* was found below the limit of quantification (<2 log CFU/g) during production. In group B (*S. aureus* inoculated), *S. aureus* showed little growth (0.48 log unit) after 24 h of fermentation. An increase in the number of *S. aureus* (0.82 log unit) was also observed after 48 h of fermentation. After 72 h, an increase in the number was not determined. Since *S. aureus* is an acid sensitive microorganism, the rate and degree of acidification during fermentation play an important role in inhibition of this microorganism (Kaban and Kaya 2006; Lücke 1998; Wang et al. 2018; Yılmaz Topcam et al. 2024). In the first 24 h of fermentation, no decrease in mean pH (5.83) was observed in group B, and even a higher pH compared to the initial pH was observed. The pH increase in this group, which did not include autochthonous strains, is thought to be related to the proteolytic activities of microorganisms in the meat environment. After 48 h of fermentation, there was a slight drop in mean pH (5.6). Despite this, the number of *S. aureus* continued to increase. At the end of 72 h, the average pH value was determined to be 5.42 and no significant increase in the number was observed. Similar results were observed in previous studies on sucuk (a type of dry fermented sausage) (Erol and Hildebrant 1992; Kaban and Kaya 2006). In contrast, as can be seen in Figure 3, in the HTS group (C, D and E) inoculated with *S. aureus*, the autochthonous strains inhibited the growth of this food-borne pathogen. These results indicated that the autochthonous strains used starter cultures inhibited the *S.aureus* by lowering the pH value during fermentation. These findings indicated observed that the acid formation during fermentation has a very important hurdle effect in HTS. In studies about Pepperoni and Geno sausage (Raccahch 1981), sucuk (Yılmaz Topcam et al. 2024) and other fermented sausage varieties (Gonzalez-Fandos et al. 1996, 1999; Marcy et al. 1985; Sameshima et al. 1998; Campaniello et al. 2020; Tangwatcharin et al. 2020), LAB has been reported to play an important role in the inhibition of *S. aureus*.

In the present study, the temperature in heat treatment stage was gradually increased, and the cooling stage was started when the internal temperature was 68 °C. With the heat treatment application (core temperature 68 °C), the number of *S. aureus* decreased below the detectable limit in all groups. *S. aureus* is a heat treatment sensitive microorganism. The D value of this microorganism is approximately 1 min at 65 °C (Lücke 1998).

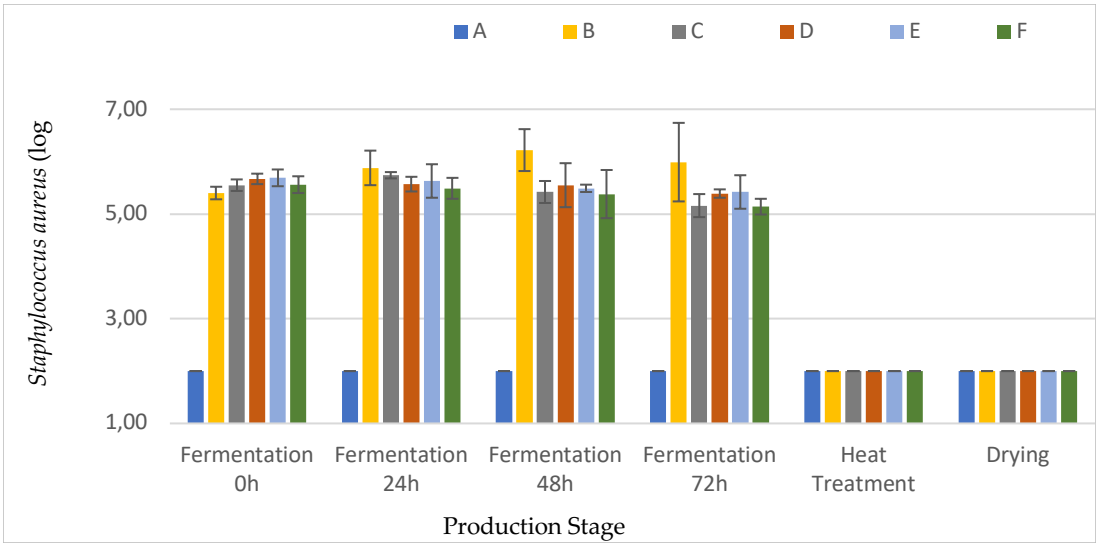

Figure 3. The changes in the *S. aureus* of HTS during the production (A: *S.aureus* and starter culture not inoculated, B: *S.aureus*, C: *S.aureus* / *L.plantarum*, D: *S.aureus* / *L.sakei*, E: *S.aureus* / *P.acidilactici*, F: *S.aureus* / *L.plantarum* / *L.sakei* / *P.acidilactici*).

3.3. Lactic Acid Bacteria and Micrococcus/Staphylococcus

The use of autochthonous LAB showed a very significant impact on LAB of sausages ($p < 0.01$). Fermentation time showed also similarly effect on LAB ($p < 0.01$). The initial LAB numbers of A and B groups were found to be 2.56 ± 0.73 and 3.09 ± 0.46, respectively. The initial LAB numbers varied between 7.22 ± 0.15 and 7.96 ± 0.14 in the groups (C, D, E and F) with autochthonous strains. After 24 h of fermentation, LAB

number increased to about 9 log CFU/g in samples contained autochthonous starter cultures, but remained at about 5 log CFU/g in A and B groups with no starter culture. The initial stage of fermentation is very important for the inhibition of *S. aureus*. As in group B, *S. aureus* could grow due to slow acidification by spontaneous LAB. According to these results, both autochthonous strains and mixtures of these strains cause good acidification during the fermentation stage and inhibit the growth of *S. aureus*. With the heat treatment applied after the fermentation stage, a significant reduction in LAB numbers was achieved (<2 log CFU/g) (Figure 4).

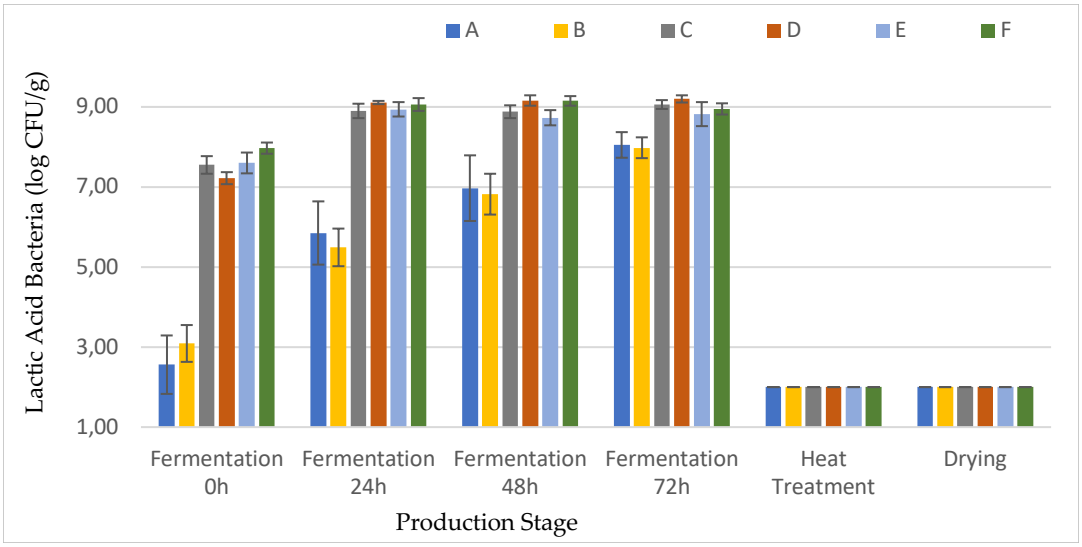

Figure 4. The changes in the LAB of HTS during the production (A: *S.aureus* and starter culture not inoculated, B: *S.aureus*, C: *S.aureus* / *L.plantarum*, D: *S.aureus* / *L.sakei*, E: *S.aureus* / *P.acidilactici*, F: *S.aureus* / *L.plantarum* / *L.sakei* / *P.acidilactici*).

In the present study, autochthonous strains had a very significant effect on *Micrococcus/Staphylococcus* number of HTS samples ($p < 0.01$). The changes in the *Micrococcus/Staphylococcus* of HTS during the production are shown in Figure 5. In the group A, the number of *Micrococcus/Staphylococcus* increased significantly due to the slowly decrease in pH. The initial number, which was about 4 log CFU/g, increased by about 2 log units after 72 hours of fermentation. Similarly, the number of *Micrococcus/Staphylococcus* increased as the fermentation time progressed in the B group contaminated only with *S. aureus*. In the presence of autochthonous LAB the number of *Micrococcus/Staphylococcus* did not show any significant change during fermentation. Morever, after heat treatment, the significant reductions were observed in all groups (<2 log CFU/g). No significant change was observed during the drying stage (Figure 5).

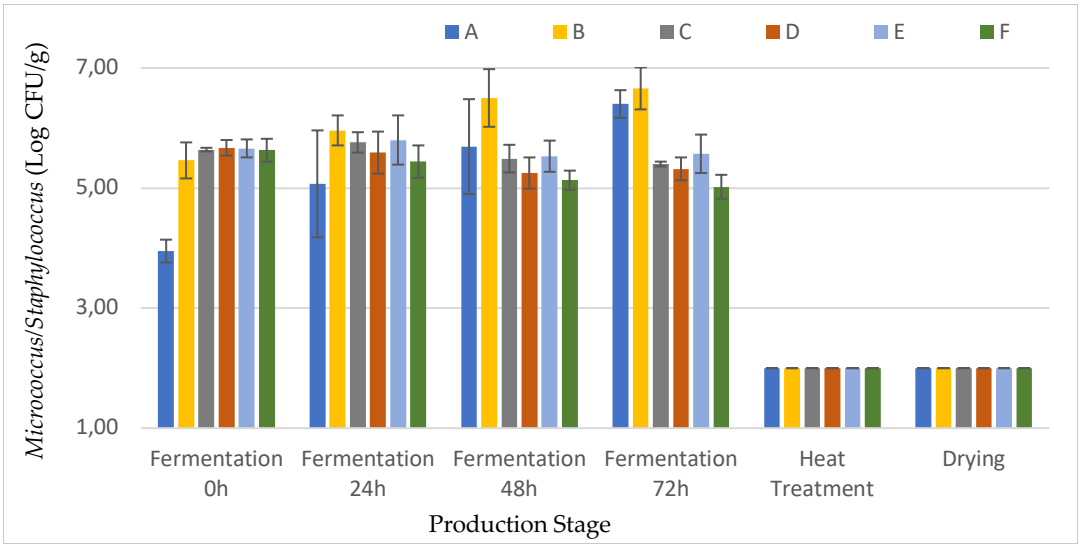

Figure 5. The changes in the *Micrococcus/Staphylococcus* of HTS during the production (A: *S.aureus* and starter culture not inoculated, B: *S.aureus*, C: *S.aureus* / *L.plantarum*, D: *S.aureus* / *L.sakei*, E: *S.aureus* / *P.acidilactici*, F: *S.aureus* / *L.plantarum* / *L.sakei* / *P.acidilactici*).

3.4. Enterotoxin Formation

In order to determine the enterotoxin production ability of *S. aureus* under production conditions of HTS, sausage groups contaminated with *S. aureus* were subjected to enterotoxin analysis using the VIDAS method. In the group B, *S. aureus* reached around 10^6 CFU/g during fermentation stage. However, no enterotoxin was detected in the final product. In another HTS groups with starter culture (C, D, E and F groups), no enterotoxin was also detected. In previously studies, it has also been reported that enterotoxin may not always be formed at high numbers of *S. aureus* (Bang et al. 2008; Notermans and van Otterdijk 1985). This study indicated also that enterotoxins may not be formed under fermentation conditions of HTS, even at the high *S. aureus* number. However, it is believed that the use of lactic starter cultures was necessary to prevent or limit growth of *S. aureus* during fermentation stage of HTS.

4. Conclusions

According to the results, the decrease in pH during fermentation of HTS is very important hurdle effect for preventing the growth of *Staphylococcus aureus*. *Latilactobacillus sakei* S15 was more effective on the inhibition of *S. aureus* at early stage of fermentation, followed by *Lactiplantibacillus plantarum* S91. However, *S. aureus* number increased in the absence of autochthonous strains used starter cultures. On the other hand, no enterotoxin was detected in all groups under the processing conditions applied in the study. Besides all this, it is estimated that *S. aureus* can grow rapidly and reach high numbers at fermentation temperatures above 22 °C. Therefore, a rapid pH drop during fermentation is of great importance for the control of *S. aureus* at both low and high fermentation temperatures. For this reason, autochthonous lactic starter cultures should be used in the production of HTS.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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