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EFFECT OF ENCAPSULATED STARTER CULTURE INCLUSION AND HEAT TREATMENT ON BIOGENIC AMINES CONTENT OF SUCUK

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ABSTRACT

In this study, the effect of encapsulated starter culture (*Lactobacillus plantarum* plus *Staphylococcus xylosus*) inclusion on biogenic amines (BAs) content of sucuk was investigated comparatively in heat treated (at ~70 °C for 20 min) and fermented sucuks. The highest contents of histamine, which is known as the most toxic BA, were observed in the fermented samples including non-encapsulated (103.6 mg/kg) and encapsulated (102.3 mg/kg) starter cultures, while the lowest values were detected in the heat treated sucuks ($p < .05$), including non-encapsulated (24.2 mg/kg) and encapsulated (21.4 mg/kg) starter cultures, at the end of 45 days of storage. Based on the principal component analysis of the microbiological count and BAs content of the samples, the heat treated and encapsulated starter culture included sucuks discriminated themselves from control groups and non-encapsulated encapsulated starter culture included samples with lower histamine and tyramine contents as well as their microbiological loads.

Keywords: Encapsulation, Biogen amine, Starter culture, Heat treated, Sucuk

Introduction

Fermentation is one of the oldest food preservation methods. Meat fermentation results in lactic acid production by certain species of lactic acid bacteria (LAB) that are naturally occurring microflora on meat or are subsequently added at known levels as starter culture (Ravyts Vuyst and Leroy, 2012). Sucuk is one of the most popular traditional dry fermented meat products in Turkey (Soyer,Ertaş and Üzümcüoğlu, 2005). Starter cultures are frequently used in fermented sucuk in order to reduce fermentation time, enhance sensory quality, and improve product safety and lower cost of fermented products. LAB are preferably used as starter culture in sucuk production together with micrococci because of their acidification ability as well as proteolytic and lipolytic activities (Leroy Verluyten and De Vuyst, 2006). Among the starter cultures *Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum*, *Staphylococcus carnosus*, *Staphylococcus xylosus* and *Pediococcus pentosaceus*, generally used as alone or their binary mix in traditional sucuk production (Bozkurt and Erkmen, 2002; Dalmış and Soyer, 2008; Gençcelep Kaban and Kaya, 2007; Kaban and Kaya, 2009; Kurt and Zorba, 2010).

Biogenic amines (BAs) are organic bases with low molecular weight that widely occur at low pH in fermented foods by decarboxylation of amino acids via microbial action (Jairath Singh Dabur Rani, and Chaudhari, 2015). The role of microorganisms and other external factors on BAs formation was extensively discussed in many of reviews (Bover Cid Miguélez-Arrizado Becker Holzapfel and Vidal-Carou, 2008; Galgano Favati Bonadio Lorusso and Romano, 2009; Kaniou Samouris Mouratidou Eleftheriadou, and Zantopoulos, 2001; Claudia Ruiz-Capillas and Jiménez-Colmenero, 2004). The presence of BAs in foods is important for several reasons such as the level of BAs is used as an indicator of quality and/or acceptability in some foods (Hernández-Jover Izquierdo-Pulido Veciana-Nogués Mariné-Font and Vidal-Carou, 1997), and high level BAs intake could cause toxic effects (Bardócz, 1995). Formation of free amino acids with proteolytic events during fermentation provides precursors for BAs. The pH level of fermented meat products has critical importance on the level of BAs. Decarboxylase activity increases with decreased pH levels thereby the production of BA increases (Bover Cid et al., 2008). One way to prevent excessive BA accumulation is inclusion of amine-negative starter culture to carry out a controlled fermentation (Bover-Cid Izquierdo-Pulido and Vidal-Carou, 2000). The most extensively studied BAs in meat and fermented meat products are tyramine, cadaverine, putrescine and histamine (Claudia Ruiz-Capillas and Jiménez-Colmenero, 2004).

In recent years, because of the great consumer awareness and developments regarding new technologies, attempts to improve the food quality with innovative techniques have become very popular. As an innovative application, encapsulation can be used to protect the sensitive biomaterials from environmental influences and provide controlled release through the semi permeable shell structure. (Corbo et al., 2016; De Prisco and Mauriello, 2016; Kailasapathy, 2002). Viability of LAB in fermented foods has been successfully improved by encapsulation (Martín Lara-Villoslada Ruiz and Morales, 2015).

Heat treated sucuk is defined as "sucuk like product" in Turkey. Manufacturers have been included a heat treatment step (at 68-70°C for 15-30 min) to sucuk production process just after filling of sucuk dough into casings. The advantages of this step include; extending shelf life, reducing of production period and production cost (Ercoşkun Tağı and Ertaş, 2010). The main aim of the heat treatment is to destroy unwanted microorganism. Meanwhile, beneficial bacteria such as LAB and Micrococcus-Staphylococcus (M-S) are also destroyed. However, development of the quality properties of fermented foods is largely dependent on maintaining the desired bacteria in an active state. To overcome such deficiencies, we used microencapsulated starter cultures including *Lactobacillus plantarum* and *Staphylococcus xylosus* in heat treated and traditional sucuks. Higher survival rate for starter cultures was achieved by encapsulation. Encapsulation procedures, morphological properties, survival rate and release behavior of starter cultures and the effect of non-encapsulated and encapsulated starter cultures inoculation on physicochemical and microbiological characteristics of heat treated and fermented sucuks were reported in our previous study (Bilenler Karabulut and Candogan, 2017). However, it was not possible to give the results of BA contents within the same article due to limited scope.

Therefore, the purpose of the present paper was to report the effect of encapsulated starter culture inclusion on BAs formation in heat treated sucuks during fermentation and storage periods, and also to compare with the formation of BAs in traditional fermented sucuk.

Materials and Methods

Starter Culture Preparation

Lactobacillus plantarum (ATCC No: 2331) and *Staphylococcus xylosus* (ATCC No: 29971) were activated and refreshed in MRS (Merck, Darmstadt, Germany) and BHI broth (Merck), respectively, at 37°C for 48h. Starter cultures in late-log phase (with the cell numbers of 3.2×10^{11} CFU/mL

and 8.4×10^{11} CFU/mL, respectively) were harvested by centrifugation at 3000 rpm for 10 min, washed in sterile saline solution (0.8% NaCl) (Bilenler Karabulut and Candogan, 2017).

Microencapsulation

Starter cultures were encapsulated according to the emulsion technique as commonly applied Sultana et al. (2000). The detailed microencapsulation process was described in the preceding paper (Bilenler Karabulut and Candogan, 2017). Sodium alginate/starch blends (Sigma-Aldrich, Steinheim, Germany) were used as wall material to encapsulate starter cultures. The highest survival rates for both encapsulated bacteria were obtained with alginate-starch blend with percentage ratios of 2:2 and 0.5:0.5 for *S. xylosus* and *L. plantarum*, respectively. The experimental materials were properly sterilized before use. Shell mixture solution was sterilized at 121°C for 15 min. After cooling to room temperature, the mixture containing 40 mL of alginate-starch and 10 mL of active cell suspension (with 11.5 log CFU/mL and 11.92 log CFU/mL, for *L. plantarum* and *S. xylosus,* respectively) were prepared. The suspension was added dropwise into 250 mL of sunflower oil containing 0.5% of Tween 80 and stirred for 20 min. The emulsion was broken by quickly adding 0.1 M $CaCl_2$ (100 mL) into the mixture while stirring. The mixture was allowed to stand for 20 min to settle alginate beads and they were washed with a solution containing 0.9 % salt and 5% glycerol. Microcapsules were harvested by low speed centrifuge at 15000 rpm for 5 min. Then the microcapsules were frozen at –18°C for 24 h and freeze-dried overnight using a freeze-dryer (Armfield, Ringwood, England). Dry microcapsules were stored at 4°C until use (Bilenler Karabulut and Candogan, 2017).

Sucuk Manufacture

Beef, lamb tail fat and additives were mixed to prepare sucuk dough according to the recipe described by Kaban and Kaya, (2009) using industrial scale equipment at a local meat factory (Malatya Meat and Meat Products) as described recently (Bilenler Karabulut and Candogan, 2017). Detail of production steps and sucuk groups with and without starter cultures in non-encapsulated or encapsulated forms are described in a flow chart (Figure 1). Non-encapsulated or encapsulated starter cultures [*S. xylosus* + *L. plantarum*] (1:1) at a level of 7 log CFU/g were used. The batter stuffed into natural casings and conditioned. Fermented sucuk groups were subjected to stepwise 14-day fermentation under the conditions given in Figure1. In heat treated sucuk production, heat treatment was carried out 24 h after stuffing in a cabin room heated conventionally at 70°C for 20 min until the internal temperature of the sucuk coils reached 70°C. Then the both sucuk groups were stored at 4ºC for 45 days. The sucuk manufacturing was replicated two times under the same production conditions. Triplicate analyses were carried out at Days 0 (after stuffing), 14 (after production), 30 and 45 during refrigerated storage.

Biogenic Amine Analysis

The extraction and derivatization of BAs were done as described by Eerol et al. (1993). Briefly, 4 g of sucuk sample was weighed to a test tube and homogenized with 10 mL of perchloric acid (70%), followed by centrifugation at 3000 rpm for 10 min. The extraction was repeated twice. Both supernatants were combined, and the final volume was adjusted to 25 mL with perchloric acid. In order to derivatization of the amines in samples, each extract was mixed with 200 µL of 2 M sodium hydroxide and 300 µL of saturated sodium hydrogen carbonate (8.5 g/100 mL pure water), then reacted with 1 mL dansyl chloride solution (10 mg/mL acetone) at 40°C for 45 min. After that, the reactant was mixed with 100 µL of 25% ammonium hydroxide and incubated at room temperature for 30 min to remove the residual dansyl chloride. The mixture was adjusted to 5 mL with 0.1 M ammonium acetate/acetonitrile (1:1) and filtered through a 0.45 µm syringe filter (Lubitech, Songjiang, China) for HPLC analysis.

Twenty microliters of the filtrate were injected into HPLC system (Shimadzu, Kyoto, Japan) equipped with a Spherisorb ODS-2 column (5 µm, 125 x 4 mm; Waters, Milford, MA, USA). The column temperature was set 25°C. The mobile phase consisted of solvent A (100% acetonitrile) and solvent B (0.01 M ammonium acetate). The flow rate was 1 mL/min. The separation was carried out by gradient elution procedure started at 50% B, and then solvent B was raised to 90% within 25 min. Individual BAs were detected at 254 nm and quantified by calibration curve prepared with seven point concentrations of authentic standards (putrescine, histamine, cadaverine, spermidine, tyramine and spermine) purchased from Sigma-Aldrich (Steinheim, Germany). BA analysis was carried out in triplicate and results of two independent batches of sucuks were expressed as mg/kg of dry matter (DM).

Statistical Analysis

The effects of starter culture inclusion in non-encapsulated or encapsulated forms and heat treatment on the BA contents of sucuk were analyzed by analysis of variance (ANOVA). Duncan's multiple-comparison test was used as a tool for comparisons of means at a level of $p < 0.05$ using the SPSS package programme version 16.0 (SPSS Inc., Chicago, IL, USA). For the principal component analysis (PCA) 24 observations (sucuk samples) and 11 variables including 6 BAs and bacteria counts from previous study (Bilenler, Karabulut, and Candogan, 2017) were used in total. Correlation coefficients between the variables determined by the coefficient of Pearson and PCA were made by using XLStat software, version 2010.2.02 (Addinsoft, Paris, France).

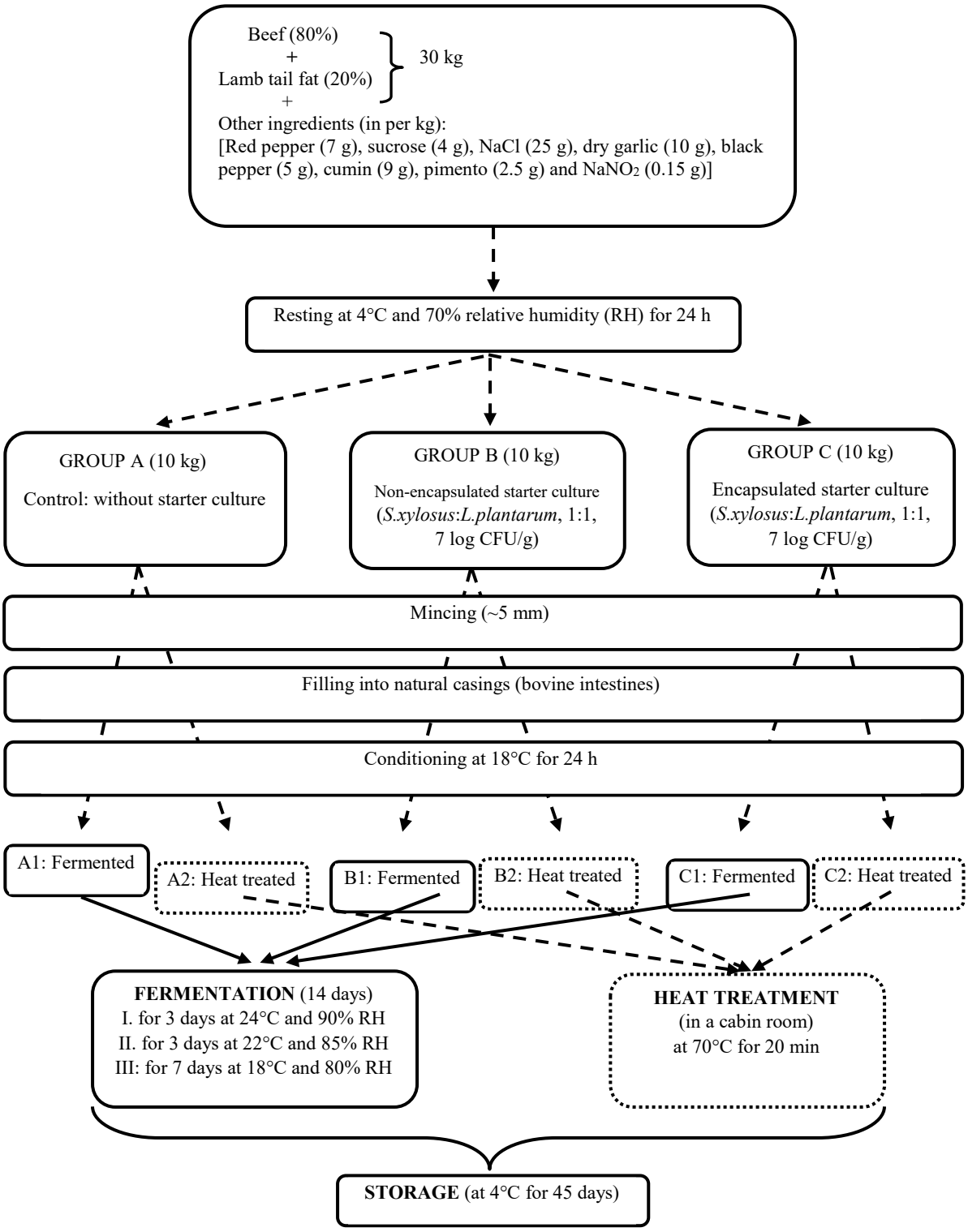

Figure 1. Flow chart for production process of the fermented and heat treated sucuks

Results and Discussion

Sucuk samples produced by fermentation or heat treatment and with/without starter culture in encapsulated or non-encapsulated forms were analyzed for their BA contents and the results are shown in Figure 2. The investigated BAs (putrescine, histamine, cadaverine, tyramine, spermidine and spermine) were detected in all sucuk samples. Histamine, tyramine and spermine were predominant amines in all sucuk samples. In general, starter culture inclusion in non-encapsulated or encapsulated forms slowed down the rate of BAs formation during fermentation and storage periods. One of the most important methods to prevent BAs formation is addition of the amine negative starter cultures to fermented meat products as reported by several reports (Bover-Cid Izquierdo-Pulido and Vidal-Carou, 1999; Gençcelep Kaban and Kaya, 2007; Papavergou, 2011; Suzzi and Gardini, 2003).

Initial amounts of BAs in control samples (A1: fermented and A2: heat treated) were higher ($p < .05$) than those of other samples at Day 0. Our previous paper (Bilenler Karabulut and Candogan, 2017) reported that control samples had significant microbial load before fermentation and heat treatment. The microbial load in these samples produced high amount of BAs during resting within 24 h (Figure 1), before fermentation process or heat treatment. This microbial flora including mainly *Enterobacteriaceae* was thought to be responsible for the high levels of BAs in control samples (A1 and A2). There is a strong relationship between the BA contents and some groups of microorganisms that are able to decarboxylate amino acids. For example, mainly putrescine and cadaverine production is a consequence of microbial activity of *Enterobacteriaceae* and tyramine production is due to the activity of Enterococci (Jairath Singh Dabur Rani and Chaudhari, 2015). In addition, the pH value of the sucuk is another factor that influences the formation of biogenic amines. Biogenic amine formation is promoted by decarboxylase activity at lower pH (Gençcelep at al., 2008; Santos 1998).

Starter culture inclusion (non-encapsulated and encapsulated forms) in both production methods [fermented (B1: non-encapsulated and C1: encapsulated) and heat treated (B2: non-encapsulated and C2: encapsulated)] affected putrescine contents at Day 0 ($p < .05$). The highest amount of putrescine contents were measured at Day 0 due to the activity of *Enterobacteriaceae.* Significant decreases were observed in putrescine contents of all samples at 14 days of storage ($p < .05$). This can be explained mainly by the competitive relationship between added starter culture and undesired microbial flora during storage days. As reported in our preceding report (Bilenler Karabulut and Candogan, 2017), starter cultures become dominant in the sucuk samples during storage. Another reason for decreasing of putrescine may be due to its conversion to spermidine. This decreasing pattern of the concentration of spermidine was reported by Rabie et al. (2014). Putrescine levels remained unchanged in the heat treated samples within 14-45 days of storage, while significant reductions were observed in the fermented group samples during this period due to higher starter culture activity. The same performance was observed with both starter culture forms (non-encapsulated or encapsulated) in reducing of putrescine and cadaverine contents. Similar reduction was observed in Spanish dry-cured "chorizo" sausage which was attributed to the lower counts of lactic acid bacteria during long storage periods (Ruiz-Capillas Jiménez Colmenero Carrascosa and Muñoz, 2007). Effect of starter cultures on reducing of putrescine and cadaverine was slightly higher in the heat treated samples (B2 and C2) than fermented ones at Day 14. On the contrary, the amount of cadaverine in fermented group was reduced faster during storage periods. Putrescine contents in all the samples decreased to about 3 mg/kg level in B1, C1, B2 and C2 samples at the end of storage period, while cadaverine reduced to 4-5 mg/kg in fermented samples and 9-10 mg/kg in heat treated samples. These amounts of putrescine and cadaverine were lower than the values reported in the literature for Turkish type sucuk (Gençcelep Kaban and Kaya, 2007), Sichuan-style sausage (Sun et al., 2016) and Italian dry fermented sausages (Tabanelli et al., 2012). Principally, putrescine and cadaverine are considered as non-toxic BAs and there are no any suggestions on their acute doses.

Among the BAs in fermented foods, histamine and tyramine are considered as the most toxic by EFSA Panel on Biological Hazards (BIOHAZ) (2011). The highest histamine level was observed in control samples (A1 and A2) at Day 0. The histamine content increased in both groups (non- and encapsulated starter culture used sucuks) after Day 14, while the increase in fermented group being higher. Histamine accumulation in fermented samples (A1, B1 and C1) continued as the storage time elapsed. Among the starter culture included sucuk samples, the highest histamine content was observed in fermented samples (103.6 and 102.3 mg/kg for B1 and C1, respectively) at Day 45, whereas the lowest ($p < .05$) was detected in heat treated samples (24.2 and 21.4 mg/kg for B2 and C2, respectively). Histamine levels in heat treated sucuks are in tolerable upper intake level recommended by EFSA who stated that intake up to 50 mg of histamine for healthy individuals can be considered safe for healthy individuals (EFSA, 2011). Histamine accumulation in heat treated samples, including non-encapsulated (B2) and encapsulated (C2) starter cultures, were not significant ($p > .05$) during fermentation and storage periods with the exception of 45 days of

storage. Histamine content in non-encapsulated starter culture used sample (B2) was higher than that of encapsulated starter culture used sample at Day 45 ($p < .05$). This can be explained by the higher number of starter cultures in C2 sample (Bilenler Karabulut and Candogan, 2017). At the end of storage period, histamine contents of encapsulated starter culture included and heat treated sucuk were considerably lower than those of reported values for different types of sausages (Bozkurt and Erkmen, 2002; Sun et al., 2016; Tabanelli et al., 2012).

Tyramine content of the samples at Day 0 was around 25 mg/kg ($p > .05$) and increased drastically to 150.20, 109.03 and 98.25 mg/kg ($p < .05$) after 45 days of storage for control, non-encapsulated starter culture used sample and encapsulated starter culture used sample in fermented group, respectively. Those levels in heat treated group were considerably low (42.61, 35.40 and 34.46 mg/kg, respectively) which may be due to destruction of the non-starter microbial flora. This effect was also observed in heat treated samples during storage period; tyramine formation within Day 30 and 45 was almost controlled ($p > .05$) in encapsulated starter culture included sample (C2). Effect of starter culture inclusion on reducing of tyramine was also confirmed in previous studies (Bover-Cid Izquierdo-Pulido and Vidal-Carou, 2000; Latorre-Moratalla et al., 2010). EFSA (2011) stated that intake up to 600 mg of tyramine can be considered safe for healthy individuals not taking monoamine oxidase inhibitor drugs. In this respect, the tyramine content of the samples may be considered within the safety ranges and considerably lower than the levels reported for Turkish dry fermented sausages (316.3 mg/kg) (Bozkurt and Erkmen, 2004), Felino-type sausages (254.38 mg/kg) (Tabanelli et al., 2012), and Italy and South Belgium fermented sausage (187 and 176 mg/kg, respectively) (Ansorena et al., 2002).

Initial amounts of spermidine for control, non-encapsulated and encapsulated starter culture included samples were 3.45, 2.29 and 2.24 mg/kg, respectively. These values increased in fermented group at Day 14 ($p < .05$) and then decreased to about 2 mg/kg during storage, while the changes in heat treated samples were not significant ($p > .05$) during fermentation and storage periods. Similar changes with spermidine were observed for spermine contents. Higher increases in fermented group after Day 14 and during storage periods indicating that heat treatment and encapsulated starter culture inclusion effectively prevented formation of these amines. As stated before, at certain levels, spermidine and spermine are not considered to be indicators of spoilage because they can be naturally exist in meats (Hernández-Jover Izquierdo-Pulido Veciana-Nogués Mariné-Font and Vidal-Carou, 1997). Kurt and Zorba (2009) reported higher level of spermidine (5.27 mg/kg) and lower level of spermine (36.35 mg/kg) in heat treated Turkish dry fermented sausage. It was reported that the concentrations of spermidine were always lower than those of spermidine due to conversion of spermidine to spermine (Hernández-Jover et al., 1997; Rabie et al., 2014).

The data were subjected to PCA on two different dataset composed of *i*) initial values (Day 0) and *ii*) fermentation and storage periods, in order to better describe the relations between factors affected by starter culture forms (non-encapsulated and encapsulated) and production methods (fermentation and heat treatment). The biplot projection of the loading and score values of the PCA for initial and fermentation-storage periods are shown separately in Figure 3 A and B, respectively. As it can be seen from Figure 3 A, 76.50 % of the total variance can be explained by the first two principal components. Control samples, starter culture forms and production methods were clearly separated on the PC1 which accounts for 57.72 % of the total variance. By looking at the orientations of the variables (loadings) and the samples (scores), it is clearly seen that the control samples discriminate themselves other sucuk samples with their higher yeast-molds and coliform counts (Bilenler Karabulut and Candogan, 2017), and higher BA contents with the exception of tyramine. As stated before, natural microbial flora probably formed a considerable amount of BAs in the sample Day 0. Among the BAs, the only putrescine correlated positively with LAB ($r = 0.204$) and M-S counts ($r = 0.149$) indicating that these microorganisms were able to generate putrescine in fermented and heat treated samples at Day 0. Negative correlation between the starter cultures and BA contents at initial stage of the production showed that there were no favorable conditions for BA formation yet. There were positive correlations between the coliforms and histamine, cadaverine, spermidine and spermine ($p < .05$) at Day 0. However, fermented and heat treated groups discriminated themselves from control samples with high counts of LAB and M-S including *L. plantarum* and *S. xylosus*, respectively.

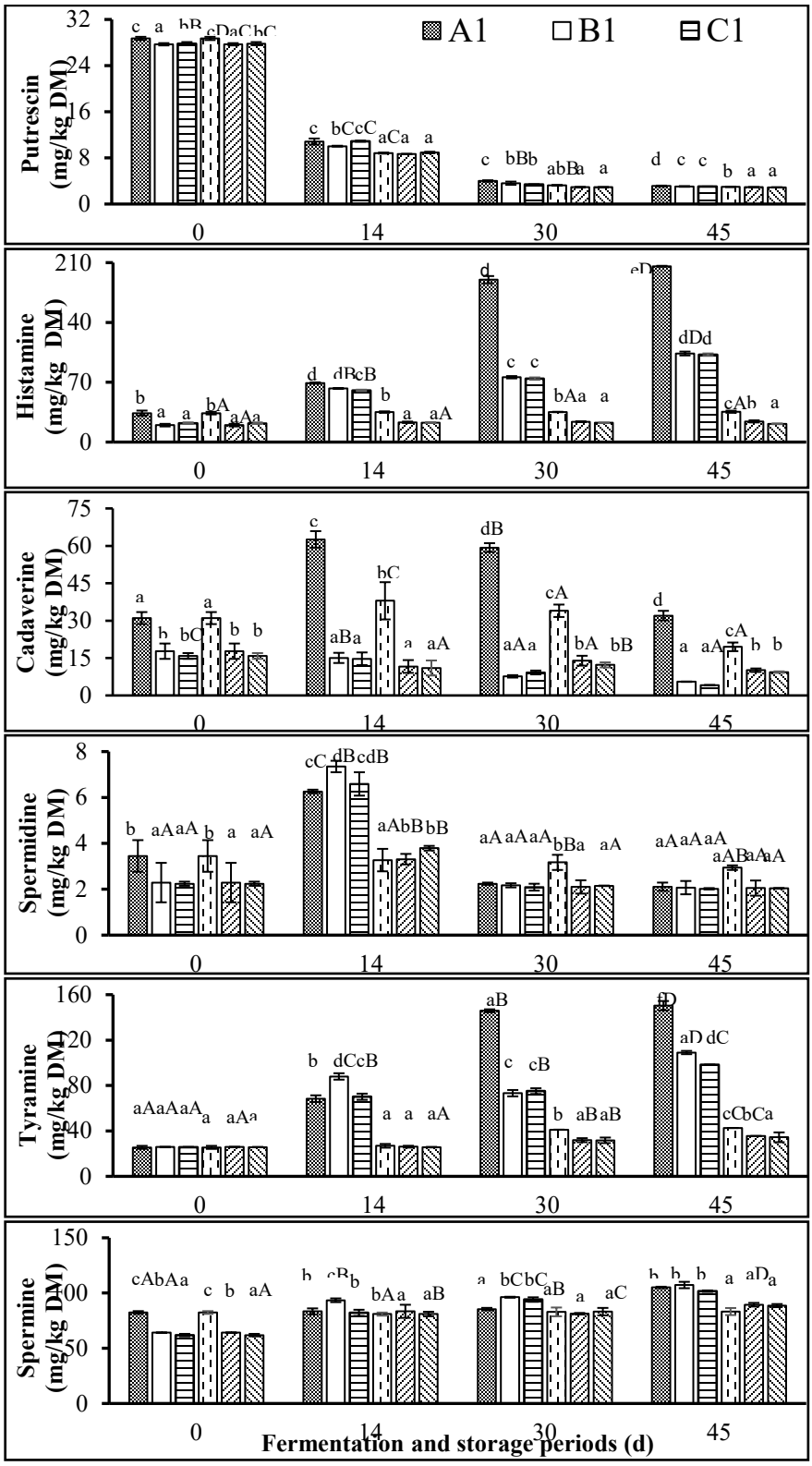

Figure 2. Changes of biogenic amines amounts during fermentation and storage periods. Different lowercase letters (a-d) in the same production day for the different sucuk samples indicate significant difference ($p < .05$). Different uppercase (A-D) between the storage days for the same sample indicate significant difference ($p < .05$)

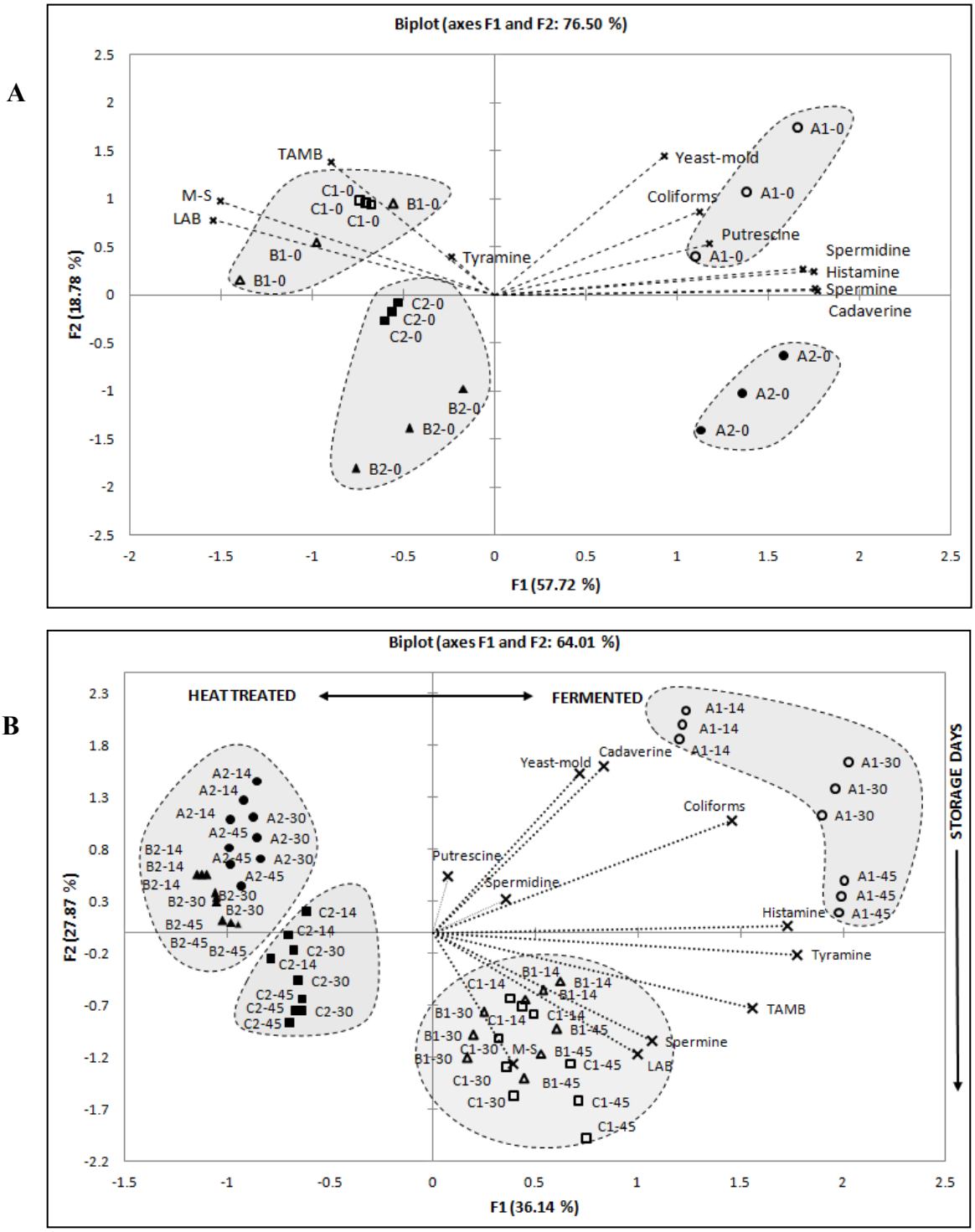

Figure 3. Biplot of the principal component analysis (PCA) carried on data of biogenic amines and microbial counts of sucuk samples just after production (A) and storage periods (B). The variables (Loadings) used for the analysis were indicated in red letters and sucuk samples (Scores) were indicated with different colors and symbols. Percentages in brackets correspond to the explained variances of the corresponding components. See Figure 1 for abbreviated sample codes.

The fermented and heat treated samples included starter culture in non-encapsulated or encapsulated forms exhibited distinct properties which was also dependent on storage periods. Figure 3 B showed the positioning of the production methods (heat treated or fermented sucuks) in the first principal plane as deducted from PCA. About 66.82 % of the total variance was explained by the first principal component. Heat treated and fermented sucuk samples clearly separated on PC1 axis. High counts of M-S and LAB, and spermine discriminated non-encapsulated and encapsulated starter culture included fermented samples. Control and non-encapsulated starter culture included samples in the heat treated group could be separated from encapsulated starter culture included sucuk in the graph due to mainly similar microbiological properties and BA contents. As given in previous study, heat treatment nearly destroyed the beneficial microbial flora as well as coliforms but not influenced survival of LAB and M-S. Correlations between the variables and observations were found to be different from that of initial stage due to biochemical changes occurred during storage period. In accordance with the literature findings (Jairath et al., 2015), coliforms were found to be primarily responsible for the formation of two most toxic BAs as well as cadaverine, since higher correlations ($p < .05$) were exist between coliforms and tyramine (r=0.637), histamine (r=0.730) and cadaverine (r=0.813). Total aerobic mesophilic bacteria (TAMB) also contributed significantly ($p < 0.05$) to the formation of the BAs with the exception of putrescine and cadaverine, while effect of M-S on BAs formation, except spermidine (r=0.308), was found to be not significant ($p > 0.05$). It should be noted that there was a high correlation between fungi (yeast-mold) and cadaverine (r=0.818), but their role is debated and, for many aspects, controversial (Gardini Özogul Suzzi Tabanelli and Özogul, 2016).

Conclusion

BAs formation in heat treated and fermented sucuks was comparatively investigated by incorporation of amine negative starter culture in non-encapsulated and encapsulated forms. Heat treatment reduced formation of BAs by destruction of amine producing microorganisms. Fermentation with encapsulated (amine negative) starter cultures reduced the presence of BAs in the heat treated product respect to the traditional fermentation. In this respect, heat treatment and encapsulated starter culture inclusion could be especially proposed for reducing histamine content of sucuk to the safer level, although any reduction would always depend on other factors influencing BAs formation.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

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