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RESEARCH ARTICLE

Influence of Sheep Tail Fat and Autochthonous Starter Culture on the Formation of Volatile Nitrosamines in Sucuk

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Abstract: The study aimed to investigate the effect of sheep tail fat (STF) on the volatile nitrosamines in a dry fermented sausage (sucuk) with/without autochthonous starter culture *(Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92). Beef fat (BF) was used as control. The production was carried out under controlled conditions with initial fermentation temperature of 22±1°C. After production, samples were subjected to pH, a_w, thiobarbituric acid reactive substances (TBARS), residual nitrite, instrumental color and nitrosamine analysis. According to results, the use of STF increased TBARS value, while it decreased L* value. The use of starter culture lowered the mean pH below 5.0. On the other hand, mean pH of 5.23 was found in the group without starter culture. Both starter culture and fat type had no significant effect on residual nitrite. N-nitrosopiperidine (NPIP) content was affected by STF. In contrast, STF showed no significant effect on N-nitrosodimethylamine (NDMA) and N-nitrosomethylethylamine (NMEA) content of sucuk. Starter culture caused an increase in NDMA levels, while decreasing N-nitrosopiperidine (NPIP). According to principal component analysis (PCA), pH, a_w, TBARS, NDMA and NPIP located on positive side of the principal component 1 (PC1), while NMEA, residual nitrite, L*, a* and b* values were in negative side of the PC1. In addition, NMEA showed more correlation with BF.

Keywords: *Lipid oxidation, Sheep tail fat, NDMA, NPIP, Sucuk, TBARS*

Kuyruk Yağı ve Yerel Starter Kültürün Sucukta Uçucu Nitrozamin Oluşumuna Etkisi

Öz: Bu çalışmada, yerel starter kültür *(Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92) içeren veya içermeyen kuru bir fermente sosiste (sucuk), koyun kuyruk yağının (KKY) uçucu nitrozaminler üzerindeki etkisinin araştırılması amaçlanmıştır. Sığır et yağı kontrol olarak kullanılmıştır. Üretim, 22±1°C başlangıç fermantasyon sıcaklığı ile kontrollü koşullar altında gerçekleştirilmiştir. Üretimden sonra örnekler pH, a_w, tiyobarbitürik asit reaktif maddeler (TBARS), kalıntı nitrit, enstrümental renk ve nitrozamin analizlerine tabi tutulmuştur. Sonuçlara göre kuyruk yağı kullanımı TBARS değerini artırırken L* değerini azaltmıştır. Starter kültür kullanılması ortalama pH'yı 5.0'ın altına düşürmüştür. Buna karşın, starter kültür kullanılmayan grupta ortalama pH değeri 5.23 olarak saptanmıştır. Hem starter kültür hem de yağ çeşidinin kalıntı nitrit üzerinde önemli bir etkisi olmamıştır. N-nitrozopiperidin (NPIP) içeriği KKY'den etkilenmiştir. Buna karşın, KKY sucuğun N-nitrozodimetilamin (NDMA) ve N-nitrozometiletilamin (NMEA) içeriği üzerinde önemli bir etki göstermemiştir. Starter kültür, NPIP'yi düşürürken NDMA düzeylerinde artışa neden olmuştur. Temel bileşen analiz sonuçlarına göre pH, a_w, TBARS, NDMA ve NPIP temel bileşen 1 (PC1)'in pozitif; NMEA, kalıntı nitrit, L*, a* ve b* değerleri ise PC1'in negatif tarafında yer almıştır. Ayrıca, NMEA, sığır et yağı ile daha fazla korelasyon göstermiştir.

Anahtar sözcükler: *Lipit oksidasyonu, Koyun kuyruk yağı, NDMA, NPIP, Sucuk, TBARS*

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Introduction

Nitrite is a multifunctional curing agent, due to with its antimicrobial and antioxidant activites, as well as its contributions to the product in terms of color and flavor, has been debated for years due to health concerns. The hazard of nitrite lies in its capability to form N-nitrosamine rather than nitrite itself [1]. N-nitrosamines are compounds formed by reactions between nitrosating agents and secondary amines. Many factors affect the nitrosamine formation in cured meat products, including ingoing nitrite levels, residual nitrite, pH, inhibitor and catalyst compounds, spice (especially black pepper), cooking and storage conditions [2,3]. Among nitrosamines, N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) are the commonly found nitrosamines in fermented sausages including sucuk [2,4]. Examining the factors affecting the formation of nitrosamines in sucuk is of great importance concerning public health. In this regard, the effect of ascorbate and black pepper on nitrosamine formation have been investigated for this product, previously [3]. Sheep tail fat can be used solely or in formulation with beef fat as raw material in sucuk production [5]. Since sheep tail fat is more susceptible to oxidation, it was thought that it may pose a risk in terms of nitrosamine formation. Some researchers have claimed that nitrosamine precursors may be formed due to lipid oxidation during ripening [6]. Free fatty acids formed through lipolysis are precursors of lipid oxidation. Secondary metabolites such as aldehydes, ketones and alcohols are formed during lipid oxidation and among these metabolites, malondialdehyde (MDA) which is measured by thiobarbituric acid reactive substances (TBARS) test, is considered as an indicator of lipid oxidation [7]. Kurechi et al.[8] reported that in vitro conditions, MDA significantly affects nitrosamine formation and that this effect is related to pH. The same researchers also stated that nitrosamine formation decreased at pH:3 and increased between pH:4-7 in MDA present, depending on the concentration of aldehyde. However, research into the interaction between lipid oxidation and nitrosamine formation is still needed. In the study, it was aimed to investigate the effect of sheep tail fat on nitrosamine formation in sucuk in the presence or absence autochthonous starter culture. In addition, the relationship between physicochemical properties and nitrosamine levels of sucuk samples belonging to both groups was determined.

Material and Methods

Material

Lean beef, and beef fat (intermuscular fat) or sheep tail fat were used as raw material. After visible fat and connective tissue were removed from lean beef, it was cut into small pieces. Similarly, fat materials were also cut into small pieces. Then, all the raw material was vacuum packaged and separately stored at -18°C for a week.

As starter culture, authochotonous strains *(Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92) were used [5]. *L. plantarum* GM77 was grown in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) and *S. xylosus* GM92 was grown in Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) at 30°C for 24 h. *L. plantarum* GM77 was inoculated with 10^7 cfu/g, while *S. xylosus* GM92 was inoculated with 10^6 cfu/g.

Sucuk Production

Four independent sausage batches were prepared: A: Beef Fat + Lean Beef; B: Beef Fat + Lean Beef + Starter culture; C: Sheep Tail Fat + Lean Beef; D: Sheep Tail Fat + Lean Beef + Starter culture. Per kg lean beef and fat (beef fat or sheep tail fat), 0.15 g sodium nitrite, 20 g salt, 10 g garlic, 7 g red pepper, 2.5 g allspice, 9 g cumin, 5 g black pepper, 4 g sucrose and were used in the production. For each treatment, 4 kg of lean beef and 1 kg of fat were used. Sucuk batches were produced in a laboratory cutter (MADO type MTK 662, Dornhan/Black Forest). Three batches were independently prepared in different times for each treatment.

The sucuk batches were filled into collagen casings (Naturin Darm, 38 mm) using laboratory filling machine (MADO Typ MTK 591, Dornhan/Schwarzwald). The sausages were placed into a climate unit (Reich, Germany). The program was used for the ripening as following; for 1 day with a temperature of 22±1°C, relative humidity of 91±2%, and for the second day with a temperature of 20±1°C, relative humidity of 91±2%. On the following days (3^{th}, 4^{th} and 5^{th} days), the temperature was kept constant at 18±1°C, and the relative humidity was gradually reduced to 89±2%, 87±2% and 84±2%, respectively [5].

Physico-chemical Analysis

The a_w value was detected by using the a_w device (Novasina, TH-500 a_w Sprint). To determine the pH, a 10 g of sample was homogenized with 100 mL of distilled water using an Ultra-Turrax (IKA factory T 25, Germany). The pH was measured with a pH meter (ATI ORION 420, MA 02129, USA) after calibrated with 4.01 and 7.01 buffers at 22°C [5]. TBARS was detected according to the method by Kilic and Richards [9]. The results were expressed as µmol MDA/kg. Residual nitrite analysis was carried out as to the method given by NMKL [10]. The instrumental color values (L*, a*, b*) were determined by the colorimeter device (Minolta Co, Osaka, Japan) with a *C D65 illuminant, an aperture size of 8 mm and standard observed of 2° [11].

For nitrosamine analysis, ten g of sample and 1M NaOH solution was placed into a centrifuge tube, after sonication, methanol of 20 mL was added to homogenize.

Then the homogenate was centrifuged (4°C, 10.000 rpm). After filtration (glass microfiber filter, 70 mm diameter; Whatman, Maidstone, UK), 5 mL of 20% NaCl solution and 15 mL of filtrate were loaded onto a ChemElut column (20 mL unbuffered; Agilent, Santa Clara, CA). After equilibration, the column was eluted with dichloromethane. The eluent was concentrated to 1 mL with a Kuderna Danish equipment. The concentrate was evaporated using a stream of nitrogen (N-EVAP-111 evaporator, Organomation, Berlin, MA) at 40°C. Gas chromatography (GC) with mass spectrometry (MS) (Agilent 6890N/Agilent 5973; Agilent, Santa Clara, CA) was used for the detection of nitrosamines. Helium as carrier gas and DB-5MS (30 m x 0.25 mm x 0.25 µm, Agilent) as column were used in the system, and the mass spectrometer was operated in selective ion mode (SIM). N-Nitrosodipropylamine-d_{14} was used as an internal standard. The oven temperature of gas chromatography was set at 50°C for 2 min, and after that oven temperature was raised to 100°C at 3°C/min, held for 5 min, then increased to 250°C at 20°C/min. EPA 521 nitrosamine mix (Supelco, Bellefonte PA) was used for identification and quantification of volatile nitrosamines (N-nitrosodiethylamine (NDEA); N-nitrosodibutylamine (NDBA); N-nitrosomethylethylamine (NMEA); N-nitrosodipropylamine, (NDPA); NPYR; NDMA; NPIP) [12]. The results were given as µg/kg. Correlation coefficient (R^2) was determined 0.9999 for all nitrosamines. Linear range, limit of detection (LOD), limit of quantification (LOQ), linear equation, correlation coefficient (R^2), recovery and relative standard deviation (% RSD) values for nitrosamines are given in *Table 1*.

Statistical Analysis

Different fat type (sheep tail fat-STF or beef fat-BF) and autochthonous starter culture (with starter culture-"with SC" or without starter culture-"without SC") were taken as factors, and experiments were performed according to the randomised complete block design with three blocks. The results of analysis were subjected to two-way ANOVA analysis using the general linear model with SPSS version 24.0 statistical program (SPSS Inc., Chicago, IL, USA). Different fat type and starter culture were fixed factors and the replicates were random effect for all analysis. The means were compared using Duncan's multiple comparison test at the $P<0.05$ level. Means and standard errors were reported. Principal component analysis (PCA) was plotted to assess the relationships between physicochemical properties and nitrosamine levels of sucuk samples belonging to both groups. PCA was carried out with the Unscrambler software (CAMO software version 10.1).

Results

The overall effects of fat type and starter culture on the pH, a_w, TBARS, residual nitrite, color values and nitrosamine levels of sucuk are given in *Table 2*. Fat type had no significant effect on pH ($P>0.05$), on the contrary, the starter culture had a notable effect on pH ($P<0.01$). The mean a_w value of the groups with and without starter culture were 0.910 and 0.905, respectively, and the difference between groups were not significant ($P>0.05$). The mean TBARS value was higher in sucuk containing STF compared to the sucuk with BF ($P<0.05$). The starter culture had no significant effect on TBARS value ($P>0.05$). Residual nitrite was also not influenced by starter culture and fat type ($P>0.05$). Sucuk with STF had a lower mean L* value than the BF group. However, starter culture caused an increase in L* value ($P<0.05$). In addition, the starter culture increased the a* value and decreased the b* value ($P<0.05$).

As for volatile nitrosamines, the fat type had no significant effect on NDMA. However, the use of starter culture caused an increase in NDMA ($P<0.05$). Neither starter culture nor fat type affected NMEA level ($P>0.05$) *(Table*

Table 1. *Linear range, limit of detection (LOD), limit of quantification (LOQ), linear equation, recovery (%) and relative standard deviation (RSD %) values of volatile nitrosamines*

Volatile Nitrosamine	Linear Range (µg/L)	LOD (µg/L)	LOQ (µg/L)	Linear Equation	Recovery %	RSD %
NDMA	0.5-20	0.32	0.98	y=39157x-9242.9	101-104.37	4.44-6.15
NMEA	0.5-20	0.42	1.28	y=37559x-524.7	97-101.07	1.85-6.78
NDEA	0.5-20	0.44	1.34	y=37649x-637.8	94-101.36	3.06-7.42
NPYR	0.5-20	0.36	1.09	y=28751x-2272.5	95.43-103.17	2.98-7.12
NPIP	0.5-20	0.32	0.98	y=32293x-7776.8	99.73-100.83	0.95-4.56
NDBA	0.5-20	0.38	1.15	y=13616x-3859.9	96.97-99.79	1.26-5.81
NDPA	0.5-20	0.15	0.46	y=6376.7x-3529.3	97.17-99.79	0.77-5.77

NDMA: *N-nitrosodimethylamine;* ***NMEA:*** *N-nitrosomethylethylamine;* ***NDEA:*** *N-nitrosodiethylamine;* ***NPYR:*** *N-nitrosopyrrolidine;* ***NPIP:*** *N-nitrosopiperidine;* ***NDBA:*** *N-nitrosodibutylamine;* ***NDPA:*** *N-nitrosodipropylamine;* ***LOD:*** *Limit of detection;* ***LOQ:*** *Limit of quantification;* ***RSD:*** *Relative standard deviation*

Table 2. *The overall effects of fat type and starter culture on pH, a_w, TBARS, residual nitrite, color values and volatile nitrosamine levels of sucuk*

Group	n	pH	a_w	TBARS µmol MDA/kg	Residual Nitrite (mg/kg)	L*	a*	b*	NDMA (µg/kg)	NMEA (µg/kg)	NPIP (µg/kg)
Fat Type (FT)											
Beef Fat (BF)	12	4.94	0.905	5.55^b	12.10	35.74^a	12.55	9.17	0.67	0.69	1.42^b
Sheep Tail Fat (STF)	12	4.92	0.909	13.46^a	10.31	34.76^b	12.59	8.92	0.84	0.72	2.09^a
SEM		0.036	0.02	0.792	0.950	0.495	0.253	0.142	0.060	0.088	0.162
Significance		ns	ns	**	ns	**	ns	ns	ns	ns	*
Starter Culture (SC)											
Without SC	12	5.23^a	0.910	9.85	11.09	33.76^b	12.04^b	8.96	0.61^b	0.57	2.30^a
With SC	12	4.63^b	0.905	9.16	11.32	36.73^a	13.0^a	9.13	0.90^a	0.85	1.20^b
SEM		0.036	0.002	0.792	0.950	0.495	0.253	0.142	0.060	0.088	0.162
Significance		**	ns	ns	ns	**	*	ns	*	ns	**
FTxSC		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

^{a,b} *Values within a column with different superscripts differ significantly at P<0.05;* ***ns:*** *not significant: P>0.05; * P<0.05, ** P<0.01;* ***SEM:*** *Standard error of mean;* ***FT:*** *Fat type;* ***BF:*** *Beef fat;* ***STF:*** *Sheep tail fat;* ***SC:*** *Starter culture;* ***TBARS:*** *Thiobarbituric acid reactive substances;* ***NDMA:*** *N-nitrosodimethylamine;* ***NMEA:*** *N-nitrosomethylethylamine;* ***NPIP:*** *N-nitrosopiperidine*

2). The group with starter culture gave a higher mean for NMEA, but the difference between the two groups was not statistically significant (P>0.05). NPIP content was found at higher level in group containing STF (P<0.05) *(Table 2)*.

PCA was applied to evaluate the relationships between factors (BF, BF_SC, STF and STF_SC) and nitrosamines, pH, a_w, TBARS and residual nitrite *(Fig. 1)*. The principal component1 (PC1) and principal component2 (PC2) were to explain 84% and 14% of the variation, respectively *(Fig. 1)*. The groups with STF and STF_SC situated on positive part of PC1, while the groups with BF and BF_SC placed on negative part of PC1 *(Fig. 1)*. In addition, pH, a_w, TBARS, NDMA and NPIP situated on positive part in PC1, while NMEA, residual nitrite, L*, a* and b* values were in negative part for PC1 *(Fig. 1)*.

Discussion

Lactic starter culture used in this study showed a good development during fermentation, lowering the pH. Similar results have been found in other studies [3,4,13]. The most important function of lactic acid bacteria during fermentation is to produce lactic acid from the sugars added to the batches. Acid formation during fermentation contributes to product safety and provide to the development of sensory and textural properties [11,14]. Therefore, the desired drying was also achieved in the sucuk without starter culture.

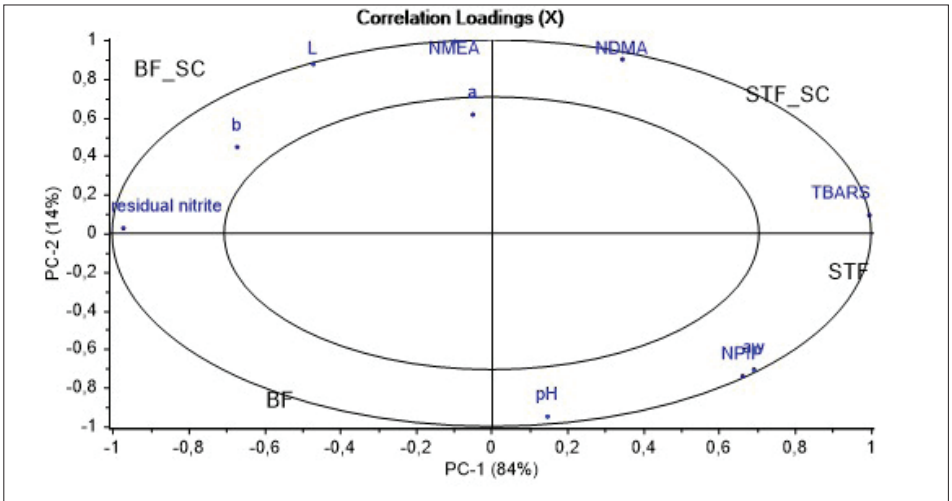

Fig 1. Principal component analysis biplot of the relationships between groups (starter culture and/or fat type) and nitrosamines (BF_SC: Beef fat with starter culture; BF: Beef fat without starter culture; STF_SC: Sheep tail fat with starter culture; STF: Sheep tail fat without starter culture)

In this study, the high TBARS value in the sucuk group containing STF is thought due to the fact that STF contains more mono- and polyunsaturated fatty acids than BF [15]. High levels of unsaturated fatty acids significantly accelerate lipid oxidation as the double bonds are easily oxidized, yielding a range of oxidation products including alcohols, ketones and aldehydes [7]. On the other hand, having no effect of starter culture on TBARS is probably due to the low pH difference between the STF and BF groups. pH also plays an important role in nitrite degradation in fermented sausages, and a decrease in pH increases nitrite degradation [11,14]. However, in the present study, although there was a significant difference between the groups with and without starter culture, no statistical difference between groups was found in terms of the residual nitrite. This result probably indicates that lowering the pH to around 5.3, as in the non-starter culture group, may be sufficient for nitrite degradation. On the other hand, starter culture addition increased a* and L* value in this study *(Table 2)*. Similar findings were also reported by Lorenzo et al.[16].

In the present study, increasing the NDMA level in sucuk group with starter culture was thought to be related with a sharp pH decrease by starter culture. The formation of volatile nitrosamine in fermented sausages can occur more easily as the pH of the product approaches optimum pH level (pH: 3.5) of the nitrosation reaction [17]. NDMA can be formed when dimethylamine reacts with the nitrosating agent [18]. In addition to pH effect, it is thought that proteolytic degradation products formed during ripening play a role in the increase of NDMA. Indeed, many researchers have stated that proteolysis degradation products are important precursors for NDMA formation [19]. Scanlan and Issenberg [20] also state that various micro-organisms produce substances that catalyze nitrosation. The mechanism of nitrosamine formation is quite complex. This mechanism is affected by many factors such as pH, oxidation-reduction potential, a_w, level of precursors, ingoing nitrite level, residual nitrite [19].

The factors examined in this study had no impact on NMEA and mean NMEA values were below than 1 µg/kg *(Table 2)*. Similar findings were reported by Herrmann et al.[2]. As seen in *Table 2*, the sucuk group containing STF gave a higher mean NPIP value. This result is linked to be due to the fact that sucuk groups produced with STF had higher TBARS values than those with BF *(Table 2)*. In studies conducted on model systems, it is also stated that MDA, an indicator of lipid oxidation, promotes the nitrosamine formation [8,21,22]. Lu et al.[23] also emphasized that lipid oxidation promotes the formation of nitrosamine. In the current study, the starter culture caused a decrease in NPIP level *(Table 2)*. This result suggested that the decrease in NPIP level by starter culture in sucuk might be associated with the reduction in its precursors.

PCA results indicate that as lipid oxidation increases, NDMA and NPIP levels increase. It also turns out that NDMA and NPIP are dependent on pH and a_w. Although the groups with starter culture (BF_SC and STF_SC) placed on the positive side of PC2, BF and STF groups placed on the negative side of PC2. These results explain that the starter culture showed a similar effect in both types of fat. Namely, PC2 separated the groups with (BF_SC and STF_SC) and without starter culture (BF and STF), regardless of fat type. On the other hand, PC1 separated the beef fat (BF and BF_SC) and sheep tail fat (STF and STF_SC) from each other. Based on these results, STF groups with and without starter culture positively affected NDMA and NPIP. In addition, NMEA more correlated with BF groups with and without starter culture *(Fig. 1)*.

In conclusion, the starter culture caused a significant decrease in pH value during fermentation of sucuk, in addition, it increased a* value. TBARS was not affected by the starter culture, but STF accelerated lipid oxidation. Addition of starter culture increased NDMA and decreased NPIP. STF significantly increased NPIP, however, NMEA was more correlated with BF. TBARS had a positive correlation with NDMA and NPIP as well as pH, a_w.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

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Ethical Statement

Ethics approval was not required for this research due to conducting *in vitro* in the laboratory.

Conflict of Interest

The author declared that there is no conflict of interest.

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