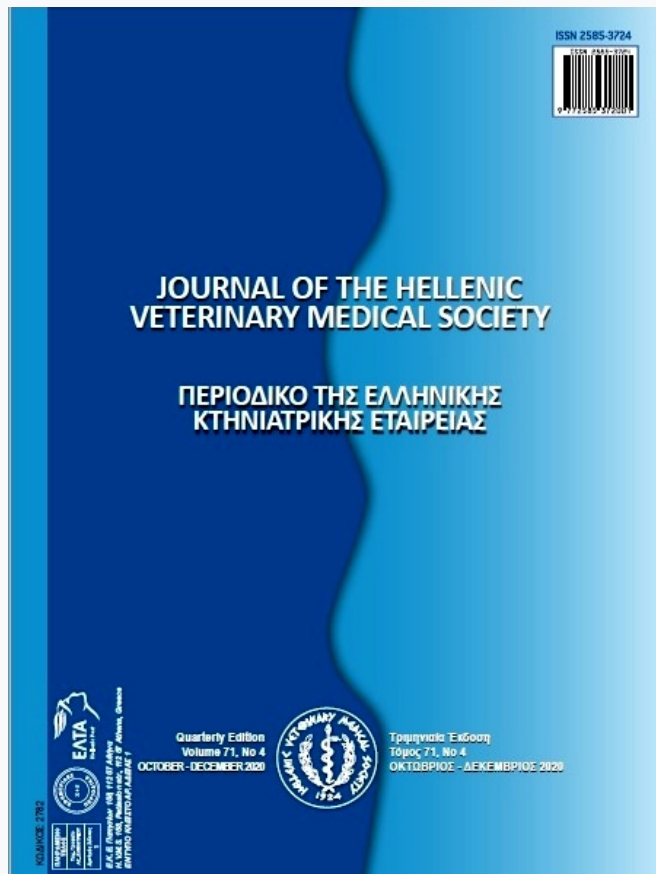


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Effects of wild type lactic acid bacteria on histamine and tyramine formation in sucuk

Y.N. Doğan¹, Ö. F. Lenger², M. Düz³, I. Doğan⁴, Z. Gürler⁵

¹Department of Veterinary, İslahiye Vocational School, Gaziantep University, Gaziantep, Turkey

²Department of Molecular Biology and Genetics, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

³Department of Chemistry, Faculty of Arts and Sciences, Afyon Kocatepe University, Afyonkarahisar, Turkey

⁴Department of Biostatistics, Faculty of Medicine, Gaziantep University, Gaziantep, Turkey

⁵Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

ABSTRACT: Biogenic amines (BAs) are formed by the decarboxylation of amino acids in fermented products and accumulate in these products due to the fermentation conditions, the natural microflora of the product, and the diversity of amino acids. Although they are inhibited by the human body, they are a hazard to public health. Starter cultures used in fermented sucuk should not have amino acid decarboxylase properties. The aim of the present study was to determine proteolytic activity, histidine and tyrosine decarboxylase enzyme activities of *Lactobacillus plantarum*, *Lactobacillus sake*, and *Lactobacillus curvatus* species and to evaluate the level of BA in sucuk groups containing these lactic acid bacteria (LAB). It was determined that none of the LAB generated these activities. While histamine values were not statistically significant in the sucuk groups ($P > 0.05$), tyramine values showed statistically significant differences ($P < 0.05$). The tyramine values of GI ($= 1.43 \pm 0.75$) and GIII ($= 2.73 \pm 1.02$) groups were lower than C ($= 8.97 \pm 5.29$) and GII ($= 7.58 \pm 2.90$) groups. According to the results of the study, *L. plantarum* or *L. curvatus* can provide more reliable fermented products with respect to tyramine formation. *L. plantarum*, *L. sake*, and *L. curvatus* could reduce histamine and tyramine formation in fermented sucuk.

Keywords: histamine, lactic acid bacteria, sucuk, tyramine

Corresponding Author:

Yağmur Nil Doğan, Gaziantep University, İslahiye Vocational School, Prof.Dr. Yavuz Coşkun Campus, Arfali, Gaziantep, 27800, Turkey
E-mail address: yagmurmildogan@hotmail.com

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INTRODUCTION

Biogenic amines in foods are formed by the enzymatic decarboxylation of amino acids as a result of the activities of microorganisms (Halász et al., 1994). The accumulation of BA in foods is associated with the presence of amino acids, the presence of microorganisms containing amino acid decarboxylase enzyme, and the establishment of proper condition (Ten Brink et al., 1990). However, not only proper hygiene but also technological measures must be applied to avoid biogenic amine formation (Leroy and Vuyst, 2004; Vidal-Carou et al., 2007). Available data show that fermented products are susceptible to the accumulation of biogenic amines (Montel et al., 1999; Parente et al., 2001). Putrescine, histamine, cadaverine, tyramine, phenylalanine, spermidine, and spermine are the most common BAs in sucuk. Histamine and tyramine are the most studied biogenic amines due to their physiological activities (Silla-Santos, 1996). Histamine and tyramine have not effect on the sensory properties of the products. However, numerous cases of food poisoning have been reported related to histamine and tyramine (Lehane and Olley, 2000; Coton and Coton, 2005). The main reason for the different levels of BA determined in sucuk is associated with the amino acid amounts, decarboxylase activity of the natural microbiota, changes in the biosynthesis of BA, and the quality of meat (Shalaby, 1996). So, It has been suggested that *Micrococcus*, *Staphylococcus*, *Pediococcus*, and *Lactobacillus* spp. should be included in the quality standards during the production of fermented sausages. The microorganisms which have amine decarboxylase can contaminate foods during any stage of processing or be present due to the product's microbiota (Maijala and Eerola, 1993; Bover-Cid et al., 2000). Therefore, there is interest in taking technological measures to reduce BA formation during the producing of traditional fermented sausages, such as the use of an autochthonous starter culture which lacks amino acid decarboxylase activity and is well adapted to the ecology of traditional meat fermentation (Benito et al., 2007; Villani et al., 2007). *L. sake* and *L. curvatus* play an important role during fermentation under uncontrolled conditions with their competitive properties and adaptation capacity (Hammes and Knauf, 1994). During fermentation, it is necessary to consider the BA formation capacity of microorganisms (Bover-Cid and Holzaphfel, 1999). High level of biogenic amines can cause serious health problems like headache, respiratory distress, cardiac palpitation, hypertension or hypotension, fa-

cialflushing, itching, swelling, diarrhea, vomiting, migraine headache, and several allergy-related disorders, moreover anaphylactic shock syndrome and death (Silla-Santos, 1996; Sohrabvandi et al., 2012). Therefore, the amino acid decarboxylase activities of microorganisms must be taken into consideration in studies involving the addition of new starter culture collections. This study aimed to evaluate the proteolytic activity, histidine, and tyrosine decarboxylase enzyme activities of *Lactobacillus plantarum*, *Lactobacillus sake*, and *Lactobacillus curvatus* obtained from the natural microbiota of sucuk and verify them with molecular methods and to determine the level of BA in sucuk containing these microorganisms.

MATERIAL AND METHODS

Bacterial Strains

Twenty-two lactic acid bacteria that were isolated and identified from traditionally produced sucuk as *Lactobacillus plantarum*, *Lactobacillus sake*, and *Lactobacillus curvatus* were used in the study (Demirel and Gürler, 2016). *Lactobacillus* 30a ATCC 33222, *Lactobacillus brevis* ATCC 367 were used as positive control strains for histidine decarboxylase and tyrosine decarboxylase respectively. Proteolytic efficiency was determined according to the methods of Lee and Simard (1984) and Franciosi et al. (2009). The method of Mangia et al. (2013) was applied for the decarboxylase activities with the modified medium content of Maijala (1993) (Table 1).

Table 1. Decarboxylase Agar Content

Content	Quantity (L)
Tryptone	5 g
Yeast Extract	4 g
Meat Extract	8 g
Tween 80	0.5 g
MgSO ₄	0.2 g
MnSO ₄	0.05 g
FeSO ₄	0.04 g
CaCO ₃	0.1 g
Amino Acid	20 g
Brome Creosol Purple	0.06 g
Agar	20 g

Confirmation of Decarboxylase related DNA by Polymerase Chain Reaction

DNA extraction was carried out according to the manufacturer's instructions (Thermo Scientific, K0721). Polymerase Chain Reaction (PCR) amplification of DNA samples was performed according to the method of Marcobal et al. (2005). The PCR was

performed in a 25 µl amplification reaction mixture containing 20 mM Tris-HCl, pH8.0, 50 mM KCl; 2.5 mM MgCl₂; each 200 µM dNTP; 1 µM primers, 1U Taq polymerase and 12.5 ng target DNA (Marcobal et al., 2005).

Marcobal et al. (2005), Coton and Coton (2005) methods were modified and used for amplification. The reactions were carried out in a Thermal Cy-

cler(Thermal Cycler, Biocycler, TC-S, Programmable thermostat)using the following cycling parameters: 5 min for the first denaturation at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 1min at 72 °C, and a final extension step of 7 min at 72 °C. The primer pairs used in the amplification process are shown in Table 2. Amplified products were examined on a 1.8% agarose gel with stained ethidium bromide.

Table 2. Oligonucleotide Primer pairs used in the PCR method for LAB

Target Gene ^a	Primer Sequence(5'-3')	Amplicon size (bp)	Reference
hdc	F: AGATGGTATTGTTTCTTATG R: AGACCATACACCATAACCTT	367 bp	Marcobal et al. (2005)
tyrdc	F: GCATACCAGAGTCCCTCAAG R: CGGATACGGACGCACAATTG	906 bp	Lucas et al. (2003)

^ahdc, histidine decarboxylase; tyrdc, tyrosine decarboxylase

Inoculation of Lactic Acid Bacteria on Sucuk and Sucuk Production

L. plantarum, *L. sake*, and *L. curvatus* which did not show proteolytic, histidine, and tyrosine decarboxylase activities verified with molecular method were selected for sucuk production. Fresh cultures were prepared using physiological saline dilutions until the desired McFarland turbidity was achieved (Öztürk, 2013).After the degree of McFarland turbidity was determined to correspond to 10⁷-10⁸ cfu/g, microorganism suspensions were prepared and added to the sucukbatter.The LAB groups are given in Table 3.

Table 3. The lactic acid bacteria groups.

Groups	The combination of starter culture
Control (C)	Non starter culture
Group I (GI)	Sucuk Batter + <i>Lactobacillus plantarum</i>
Group II (GII)	Sucuk Batter + <i>Lactobacillus sake</i>
Group III (GIII)	Sucuk Batter + <i>Lactobacillus curvatus</i>

The formulation and method indicated by Gökalp et al. (2004) was modified and used for sucuk production. The additive ratios are shown in Table 4. Maturation conditions were applied according to the method of Kaban and Kaya (2007).

Table 4. The additives and their proportions

The additives	The ratios (%)
Salt	1.6
Sugar	0.4
Garlic	1
Red pepper	1.4
Black pepper	0.4
Cumin	0.9
Pimento	0.1

Measurement of Biogenic Amines

BAs were measured by the method of Köse et al. (2011). According to the method, 5 g of the sample was taken and 50 mL of 0.1 M hydrochloric acid was added. The homogenized mixture was centrifuged at 4000 rpm at 4°C for 20 minutes. After the supernatant was removed, 100 µL of 2 N sodium hydroxide, 150 µL of saturated sodium bicarbonate, and 1 ml of dansyl chloride were added. The mixture was incubated at 40°C for 45 minutes. After incubation, it was kept at room temperature for 10 minutes. 50 µl of 25% NH₃ was added and left to stand for 30 minutes at room temperature. Finally, 5 mL of ammonium acetate: acetonitrile was added and passed through a 0.45 µm filter and injected into the HPLC system(Shimadzu Prominence, ACE5 C-18 (250 x 4,6 mm, 5 µm)) (Köse et al., 2011). Histamine and tyramine analysis of the sucuk samples were carried out in Burdur Mehmet Akif Ersoy University Scientific and Technology Application and Research Center, Turkey. The analyses were repeated three times. The mean of three measurements was used in statistical analysis.

Statistical Analysis

The normality of data was verified using the Shapiro-Wilk test ($P > 0.05$). One-Way ANOVA test was used to compare the variables according to the experimental groups. Moreover, the LSD post-hoc test was used at a 5% significance level. The analyses were conducted with SPSS 22.0 program.

RESULTS

None of the LAB isolates (12, 6, and 4 of them were *L. plantarum*, *L. sake*, *L. curvatus*, respectively) displayed proteolytic, histidine, and tyrosine decar-

boxylase activities with culture methods. Subsequently, the negative activities of the isolates were verified by PCR. The amounts of histamine and tyramine obtained from the HPLC analysis are given in Table 5. When histamine and tyramine values were compared according to sucuk groups, it was determined that differences in the histamine values were not statistically significant ($P > 0.05$). On the other hand, the differences in tyramine values among the sucuk groups were statistically significant ($P < 0.05$). Accordingly, the tyramine values of GI ($= 1.43 \pm 0.75$) and GIII ($= 2.73 \pm 1.02$) groups were lower than C ($= 8.97 \pm 5.29$) and GII ($= 7.58 \pm 2.90$) groups.

Table 5. Comparison of histamine and tyramine values according to sucuk groups

BA		N	Mean \pm SD	F	P
Histamine (mg/kg)	C	3	11.42 \pm 5.82	1.818	0.222
	GI	3	15.08 \pm 4.94		
	GII	3	7.17 \pm 1.51		
	GIII	3	9.41 \pm 3.71		
Tyramine (mg/kg)	C	3	8.97 \pm 5.29 ^a	4.227	0.046*
	GI	3	1.43 \pm 0.75 ^b		
	GII	3	7.58 \pm 2.90 ^a		
	GIII	3	2.73 \pm 1.02 ^b		

Note: Values are the mean of triplicate measurements \pm standard deviation; * $P < 0.05$; a,b: The different letters indicate the difference between the groups; N; Number of analysis.

DISCUSSION

The phenotypic properties of LAB may vary during fermentation, geographical conditions, and the origin of the isolates. It has been recommended to use molecular methods in LAB researches since false positive or false negative results can be obtained by culture methods. In this study, biogenic amine genes have been confirmed by molecular methods. However, the same species of LAB may also be positive for amino acid decarboxylase. While Choudhury et al. (1990), De Llano, (1998), Leuschner and Hammes (1998), Bover-Cid and Holzaphfel (1999) submitted tyrosine decarboxylase of certain LAB; Dapkevicius et al. (2000), Maijala (1994), Bover-Cid and Holzaphfel (1999) reported histidine decarboxylase activity. Moreover, Straub et al. (1995) declared both histidine and tyrosine decarboxylase activity by culture methods. On the other hand, Maijala, (1993); Silla-Santos (1998); Montel et al. (1999); Bover-Cid et al. (2001) did not find histidine decarboxylase activity. Although Constantini et al. (2006) reported that no histidine decarboxylase gene region in the LAB or tyrosine decarboxylase gene region in any of the others except for *L. brevis*, De las Rivas et al. (2008),

Ruiz-Moyano (2009), Landeta et al. (2013) reported that some LAB contained tyrosine decarboxylase gene regions in molecular studies. It has been reported that BA levels can be reduced in fermented sucuk by using non-BA starter cultures and the ensuing competition with non-starter LAB (Maijala, 1994). BA formation is an important criterion for the selection of starter culture to be used in sucuk production. In this study, it has been determined with both culture and molecular methods that none of the evaluated strains affected BA formation. However, BA formation can be observed in fermented sucuk depending on the diversity of the microbiota, the competitive properties with other LAB, and the fermentation conditions.

According to the HPLC results, *L. sake* and control group were more effective than *L. plantarum* and *L. curvatus* in terms of tyramine formation. Histamine and tyramine formation efficiency can be sorted from high to low as *L. plantarum*, *L. curvatus*, and *L. sake*; *L. sake*, *L. curvatus*, and *L. plantarum*, respectively. Various researchers (Ten Brink et al., 1990; Buncic et al., 1993; Şenöz et al., 2000; Ekici et al., 2004; Erkmen and Bozkurt, 2004) informed that histamine con-

centration varied between 1 mg/kg and 478.2 mg/kg in fermented sucuk.

The use of starter culture has decreased the histamine level in the study. However, the effect of *L. plantarum* on histamine formation may change this situation compared to the control group. This difference may be due to the poor ability of *L. plantarum* to compete with microorganisms or non-starter LABs as a result of initial contamination in the sucuk batter or during fermentation.

Erkmen and Bozkurt (2004) detected between 1.2 and 316.3 mg/kg tyramine levels in 50 sucuk samples. Although using starter culture in Turkish sucuk reduces the formation of putrescine, spermine, and histamine, it does not affect the formation of tryptamine, 1,7-diamino heptane, serotonin, and spermidine (Bozkurt and Erkmen, 2004). After maturation at 15 °C for 21 days, the level of tyramine was determined as 30 mg/kg in the sucuk samples which contained *L. curvatus* and *Staphylococcus xylosus*. Whereas, the tyramine level detected in the control group which did not contain starter culture was detected as 85 mg/kg. Şenöz et al. (2000) declared that the amount of tyramine in sucuk without starter culture was 400-617 mg/kg. Furthermore, tyramine levels in the samples with starter culture have been reported as 125-1173 mg/kg. In summary, the amount of BA in sucuk with starter culture was significantly lower than sucuk samples with non-starter culture (Şenöz et al., 2000). It has been noted that *L. sake* is more effective in the formation of tyramine than *L. plantarum* and *L. curvatus* in the study. Taking into consideration the amount of tyramine in fermented sucuk, with *L. plantarum* or *L. curvatus* as mixing cultures it can be stated that such fermented products are more reliable than with other starter cultures.

CONCLUSION

Although fermented sucuk production has been carried out using starter culture without the ability to form BA, a certain amount of BA formation which is harmful to human health is observed. The human body has a mechanism that decreases BA levels to a certain level. However, these mechanisms may not be effective under some circumstances like gastrointestinal disease, alcoholism. Therefore, control measures must be taken and monitoring systems of every production step until consumption to ensure hygienic quality, production, and storage conditions. When the relationship between the starter cultures has been ex-

amined, it has been found that *L. plantarum* and *L. curvatus*, having negative proteolytic and decarboxylase activity, significantly reduce tyramine levels. Similarly, histamine levels have been decreased by *L. sake* and *L. curvatus* in comparison to the control group. BAs are known to be food quality indicators and harmful to health. Therefore, it can be said that the use of starter culture reduces histamine and tyramine formation. Probiotic microorganisms in fermented products can be protected from environmental conditions by surrounding meat and fat. Also, they can produce bacteriocins or low molecular weight antibacterial compounds to combat pathogens and form the dominant flora. The LAB used in this study may have such an effect on biogenic amine forming microorganisms. However, further studies are needed to determine how they reduce BA formation. It has been determined that classical culture methods can give false positive results in BA decarboxylase determination. Therefore, molecular methods are recommended. The values determined in the study are below the toxic limits. However, this level does not mean that there is no risk for susceptible individuals. The activity of LAB on BA formation is particularly important for fermented foods. Biogenic amine formation factors should be identified and protective measures taken during the product development to ensure public health.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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