Evolution of amino acids and biogenic amines in traditional dry-fermented sausage Sjenički sudžuk during processing

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Abstract. The evolution of free amino acids and biogenic amines during 23 days of drying and ripening was determined in the traditional, dry-fermented sausage, Sjenički sudžuk. The concentration of most amino acids increased significantly (P < 0.05) over time, giving rise to a final concentration of total free amino acids of 600 mg/100g dry matter. Thus, the chief precursors became available for indigenous aminogenic microbiota, enabling formation and accumulation of biogenic amines. Total biogenic amines reached the level of 399 mg/kg by day 23 of processing. Putrescine and tyramine were the predominant amines. Their concentrations increased significantly (P < 0.05) during processing, ranging from 91.9 to 212 mg/kg and from 48.5 to 147 mg/kg, respectively. A positive outcome of this study is the very low registered concentration of histamine (9.69 mg/kg), the most important amine both from the toxicological and hygienic points of view.

1. Introduction
Sjenički sudžuk is dry-fermented sausage traditionally produced in a part of the Pešter plateau, an area near the town of Sjenica, southwestern Serbia. Nowadays, this sausage is produced according to original recipes, using just basic ingredients, such as beef, salt and spices, without the use of food additives or microbial starters [1].

Throughout the fermentation and ripening of dry-fermented sausages, the proteins undergo important degradation changes, resulting in generation of numerous compounds such as polypeptides, peptides, free amino acids, aldehydes, organic acids and ammonia [2, 3, 4, 5]. Accordingly, the quantity of free amino acids, the main precursors of biogenic amines, rises during processing, which in combination with the presence of decarboxylase-positive microbiota can lead to the formation and accumulation of these compounds [3, 6, 7, 8]. Biogenic amines are organic bases that are considered negative from both the safety and hygienic points of view [8, 9, 10, 11]. Thus, it is important to monitor the accumulation of biogenic amines in fermented meat products and to try to control all the factors that can contribute to this process. Consequently, the aim of this research was to study the formation and accumulation of free amino acids and biogenic amines, during smoking, drying and ripening of traditional dry-fermented sausage Sjenički sudžuk.
2. Material and methods

2.1. Sausage preparation and samples

Sjenički sudžuk were manufactured according to a traditional procedure in one small meat processing enterprise located in the town of Sjenica. Fresh boneless beef (approximately 75% lean) was salted using 35 g/kg of common salt (NaCl), and maintained at 4°C for 7 days. After this period, salted meat was ground through a 4 mm diameter mincing plate and mixed together with the other ingredients (raw garlic paste – 4 g/kg, black pepper – 3 g/kg, red sweet paprika powder – 2 g/kg), until a homogenous batter was obtained. Prepared batter was stuffed into natural casings with a diameter of approx. 40 mm and a length of approx. 50 cm. The ends of the sausages were tied off and bound together, forming a horseshoe shape. Raw sausages were entirely processed in a traditional smoking/drying room during 23 days. The environmental conditions (air temperature (°C) and relative humidity (%)) during processing are shown in Fig. 1.

For sampling, the seasoned batter prior to stuffing (0) and three randomly selected sausages were taken after 3, 7, 15 and 23 days of processing. The sausages were homogenized, vacuum packed and stored at -20°C pending analysis. Analyses for all samples were carried out in duplicate.

![Figure 1. Environmental temperature (°C) and relative humidity (%) recorded throughout processing of Sjenički sudžuk](image)

2.2. Determination of dry matter

Moisture content was quantified according to the ISO recommended standard [12], by heating the samples to 105°C until constant weight. Dry matter (dm) was calculated as the material remaining after removal of water.

2.3. Determination of free amino acids (FAA)

FAA in sausages were determined using ion exchange chromatography with utilization of Automatic Amino Acid Analyzer Biochrom 30+ (Biochrom, Cambridge, UK), according to Rabie et al. [6], with several modifications. Briefly, 20 ml of 10 % (v/v) trichloroacetic acid was added to 3 g of sample, the mixture was homogenized using a T18 Basic Ultra Turrax (IKA-Werke GmbH & Co. KG), and the extract was filtered through filter paper (FiltroTech, Fleury-les-Aubrais, France). The extracts were then centrifuged at 7000 g for 15 min using a centrifuge 5804 R (Eppendorf, Hamburg, Germany).
The supernatant was finally collected and filtered through 0.22 µm pore size PTFE filter (Plano, Texas, USA), and the filtrate obtained was transferred to an HPLC vial (Agilent Technologies, USA). FAA contents were determined by reaction with ninhydrin (Biochrom, Cambridge, UK), with photometric detection at 2 wavelengths, 570 nm and 440 nm (for proline), and expressed as mg 100 g⁻¹ of dry matter.

2.4. Determination of biogenic amines (BA)
BA (tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine) were determined according to the procedure described by Ikonić et al. [4]. BA were determined as their dansyl derivatives, using liquid chromatography (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a binary pump, an online vacuum degasser, an auto sampler and a thermostated column compartment, on an Agilent, Eclipse XDB-C18, 1.8 µm, 4.6 × 50 mm column. Solvent gradient was performed by varying the proportion of solvent A (acetonitrile) to solvent B (water) as follows: initial 50% B; linear gradient to 10% B in 7.6 min, 10% B to 10 min; linear gradient to 50% B in 2 min. The system was equilibrated 3 min before each analysis. Flow rate was 1.5 mL/min, column temperature was 40°C and 5 mL of sample was injected. BA contents are expressed as mg kg⁻¹ of sample.

2.5. Statistical analyses
One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 13.3 (TIBCO Software Inc., Palo Alto, Ca, USA). Differences were considered significant at $P < 0.05$.

3. Results and discussion
Changes in the concentration of FAA during the processing period are depicted in Table 1. The total FAA concentration in the raw sausage batter was 388 mg/100g dm. Throughout 23 days of drying and ripening, an increase in the concentration of most amino acids was registered, giving significant rise ($P < 0.05$) to a final concentration of total FAA of 600 mg/100g dm. The amino acids that primarily contributed to this increase during ripening were glutamic acid, leucine and valine followed by threonine, lysine and phenylalanine. Gradual release of amino acids during ripening is characteristic in dry-fermented sausages. The registered increasing trend of FAA in Sjenički sudžuk is in accordance with previously reported findings by a number of authors [2, 5, 6]. On the contrary, the concentrations of serine, tryptophan and arginine decreased significantly ($P < 0.05$), which could indicate more intense uptake and conversion to BA of these compounds by bacteria, than their production during ripening [2, 6].

The predominant FAA in Sjenički sudžuk were glutamic acid (60.8-150 mg/100g dm), alanine (69.8-81.8 mg/100g dm), serine (109-81.6 mg/100g dm) and leucine (16.8-55.1 mg/100g dm). In sum, they accounted for about 65% of the total FAA, both in the raw sausage batter and in dried sausage. These results confirm previous reports regarding the highest prevalences of glutamic acid [13] and alanine [14] in fermented sausages. Also, they differed partly from results obtained by Rabie et al. [6], who found the highest concentration of alanine, aspartic acid and glycine in beef sausage after 28 days of storage, and those reported by Domínguez et al. [5] on non-started (i.e., no starter culture) dry-fermented foal sausage, who observed leucine, cysteine and phenylalanine as the chief FAA.

Lysine, histidine, arginine, phenylalanine and tyrosine are the main precursors of dietary BA, tyramine, putrescine, cadaverine, histamine and phenylethylamine [6, 7]. Due to proteolytic changes, all of these BA, except tyrosine, significantly increased ($P < 0.05$) during drying and ripening of Sjenički sudžuk (Table 2). FAA availability in combination with activity of decarboxylase-positive microbiota can lead to formation and accumulation of BA in fermented sausages [3, 6, 7, 8]. This fact also appears to hold true in the case of Sjenički sudžuk. As can be seen from Table 2, the concentration of total BA ranged from 0 to 399 mg/kg, and this was lower than the maximum threshold of 1000 mg/kg, which is considered dangerous for human health [4, 6, 9]. The relatively low level of total BA
detected during the processing period was most likely the consequence of unfavorable conditions for growth and activity of aminogenic microbiota (low temperature; Fig. 1) [3, 4].

Table 1. Changes in concentration (mg/100g dm) of individual and total free amino acids in *Sjenički sudžuk* during processing (mean ± standard deviation)

| Amino acid     | 0      | 3      | 7      | 15     | 23     |
|----------------|--------|--------|--------|--------|--------|
| Aspartic acid  | 7.27 ± 0.58<sup>a</sup> | 6.85 ± 0.31<sup>a</sup> | 9.94 ± 1.48<sup>b</sup> | 13.8 ± 2.16<sup>b</sup> | 15.0 ± 3.73<sup>b</sup> |
| Threonine      | 7.54 ± 0.64<sup>b</sup> | 12.6 ± 3.04<sup>b</sup> | 19.2 ± 6.48<sup>c</sup> | 23.5 ± 0.75<sup>a</sup> | 25.4 ± 1.59<sup>a</sup> |
| Serine         | 109 ± 12.8<sup>a</sup> | 104 ± 3.64<sup>a</sup> | 96.5 ± 6.48<sup>ac</sup> | 83.7 ± 1.38<sup>b</sup> | 81.6 ± 7.93<sup>b</sup> |
| Glutamic acid  | 60.8 ± 5.03<sup>b</sup> | 86.9 ± 6.59<sup>c</sup> | 109 ± 6.35<sup>d</sup> | 141 ± 8.71<sup>a</sup> | 150 ± 8.12<sup>a</sup> |
| Proline        | 3.92 ± 0.30<sup>c</sup> | 11.2 ± 0.43<sup>a</sup> | 11.1 ± 0.56<sup>a</sup> | 12.5 ± 1.10<sup>b</sup> | 12.4 ± 0.19<sup>b</sup> |
| Glycine        | 11.3 ± 0.25<sup>b</sup> | 12.8 ± 1.43<sup>b</sup> | 15.9 ± 1.50<sup>a</sup> | 17.8 ± 1.41<sup>a</sup> | 18.9 ± 2.80<sup>a</sup> |
| Alanine        | 69.8 ± 6.02<sup>a</sup> | 75.3 ± 10.3<sup>a</sup> | 79.5 ± 7.52<sup>a</sup> | 82.4 ± 9.06<sup>a</sup> | 81.8 ± 3.92<sup>a</sup> |
| Cysteine       | 16.9 ± 0.64<sup>a</sup> | 18.4 ± 3.33<sup>a</sup> | 20.2 ± 2.42<sup>a</sup> | 21.7 ± 4.13<sup>a</sup> | 22.9 ± 4.03<sup>a</sup> |
| Valine         | 12.7 ± 2.38<sup>a</sup> | 17.0 ± 1.40<sup>a</sup> | 25.5 ± 2.04<sup>a</sup> | 32.1 ± 2.45<sup>b</sup> | 34.7 ± 4.12<sup>b</sup> |
| Methionine     | 9.12 ± 0.97<sup>c</sup> | 10.1 ± 1.71<sup>a</sup> | 13.4 ± 1.98<sup>ab</sup> | 15.9 ± 4.47<sup>b</sup> | 16.4 ± 1.16<sup>b</sup> |
| Isoleucine     | 11.9 ± 0.99<sup>c</sup> | 11.5 ± 0.90<sup>a</sup> | 16.6 ± 2.90<sup>b</sup> | 20.1 ± 4.23<sup>b</sup> | 21.3 ± 0.79<sup>c</sup> |
| Leucine        | 16.8 ± 0.50<sup>a</sup> | 25.6 ± 5.56<sup>a</sup> | 41.4 ± 7.43<sup>a</sup> | 51.6 ± 3.66<sup>b</sup> | 55.1 ± 4.42<sup>b</sup> |
| Tyrosine       | 1.33 ± 0.50<sup>a</sup> | 1.57 ± 0.62<sup>a</sup> | 1.73 ± 0.83<sup>a</sup> | 1.61 ± 0.56<sup>a</sup> | 1.61 ± 0.36<sup>a</sup> |
| Phenylalanine  | 3.43 ± 0.91<sup>a</sup> | 6.52 ± 1.24<sup>a</sup> | 12.6 ± 1.79<sup>a</sup> | 16.4 ± 2.99<sup>b</sup> | 17.5 ± 2.58<sup>b</sup> |
| Histidine      | 5.61 ± 0.30<sup>b</sup> | 6.49 ± 0.69<sup>b</sup> | 9.40 ± 0.99<sup>a</sup> | 10.8 ± 2.83<sup>a</sup> | 11.1 ± 0.94<sup>a</sup> |
| Tryptophan     | 10.7 ± 0.52<sup>c</sup> | 8.49 ± 0.57<sup>d</sup> | 3.81 ± 0.20<sup>c</sup> | 1.92 ± 0.14<sup>b</sup> | 0.50 ± 0.19<sup>a</sup> |
| Lysine         | 14.6 ± 1.13<sup>a</sup> | 13.1 ± 0.90<sup>a</sup> | 22.0 ± 2.92<sup>c</sup> | 28.0 ± 3.75<sup>b</sup> | 30.6 ± 2.58<sup>b</sup> |
| Arginine       | 15.0 ± 2.85<sup>b</sup> | 15.0 ± 1.97<sup>b</sup> | 8.03 ± 0.78<sup>c</sup> | 3.07 ± 0.54<sup>a</sup> | 3.94 ± 0.42<sup>a</sup> |
| Total AA       | 388 ± 11.2<sup>b</sup> | 443 ± 5.54<sup>d</sup> | 516 ± 40.6<sup>d</sup> | 578 ± 45.7<sup>a</sup> | 600 ± 1.16<sup>c</sup> |

*Means within the same row not followed by common letters differ significantly (P < 0.05)*

Putrescine was the most abundant amine found in *Sjenički sudžuk*. The putrescine concentration increased significantly (P < 0.05) during the ripening period, ranging from 91.9 to 212 mg/kg. This result is in accordance with previous reports regarding putrescine concentration in dry fermented sausages [6, 15]. Conversely, the putrescine level found in *Sjenički sudžuk* is higher than those determined in non-started sausages by Latorre-Moratalla et al. [7] and Domínguez et al. [5], and lower than levels obtained by Roseiro et al. [3] in Portuguese traditional, dry-fermented sausage. Putrescine concentration can be used as an indicator of raw material and/or manufacturing practice hygiene, since its accumulation is related to the activity of contaminant bacteria, such as *Enterobacteriaceae* [3, 8].

Tyramine was the second most common amine found in *Sjenički sudžuk* (147 ± 8.30 mg/kg) after 23 days of drying and ripening, confirming previously reported findings regarding its high abundance in dry-fermented sausages. Tyramine concentration is closely related to lactic acid fermentation, due to the high potential of many lactic acid bacteria for tyrosine decarboxylation [3, 8, 11]. This amine is directly influenced by the level of tyrosine, which remained essentially constant (Table 1), indicating that on release, this amino acid was used for metabolic reactions by the sausage microbiota, and formation of tyramine resulted [6, 7].

With respect to histamine, the most important BA from the toxicological and hygienic aspects, a slight accumulation was registered after 23 days, amounting 9.69 mg/kg. Thus, the concentration of histamine in *Sjenički sudžuk* was much lower than its allowable limit in food (100 mg/kg) [5, 6, 8],
and much lower than the level reported by EFSA [16] for European fermented sausages (approx. 25 mg/kg).

Moreover, the sum of vasoactive amines (histamine, tyramine, tryptamine, phenylethylamine) did not exceed 200 mg/kg, indicating good hygienic conditions and application of good manufacturing practice during the processing of this Šjenički sudžuk [10].

Table 2. Changes in concentration (mg/kg) of individual and total biogenic amines in Šjenički sudžuk during processing (mean ± standard deviation)

| Biogenic amine          | Processing time (day) | 0      | 3      | 7      | 15     | 23     |
|-------------------------|-----------------------|--------|--------|--------|--------|--------|
| Tryptamine              | ND                    | ND     | ND     | ND     | ND     | ND     |
| Phenylethylamine        | ND<sup>a</sup>        | 36.0 ± 1.33<sup>b</sup> | ND<sup>a</sup> | ND<sup>a</sup> | ND<sup>a</sup> | ND<sup>a</sup> |
| Putrescine              | ND<sup>a</sup>        | ND<sup>a</sup> | 91.9 ± 6.24<sup>b</sup> | 190.4 ± 0.98<sup>c</sup> | 212 ± 10.1<sup>d</sup> |
| Cadaverine              | ND<sup>a</sup>        | ND<sup>a</sup> | 20.4 ± 2.12<sup>b</sup> | 25.3 ± 0.68<sup>c</sup> | 30.8 ± 3.76<sup>d</sup> |
| Histamine               | ND<sup>a</sup>        | ND<sup>a</sup> | ND<sup>a</sup> | ND<sup>a</sup> | 9.69 ± 0.70<sup>b</sup> |
| Tyramine                | ND<sup>a</sup>        | 48.5 ± 2.91<sup>b</sup> | 102.8 ± 3.61<sup>c</sup> | 138 ± 3.60<sup>d</sup> | 147 ± 8.30<sup>e</sup> |
| Total BA                | ND<sup>a</sup>        | 84.4 ± 4.24<sup>b</sup> | 227 ± 12.0<sup>c</sup> | 353 ± 5.26<sup>d</sup> | 399 ± 22.9<sup>e</sup> |

<sup>a-d</sup>Means within the same row not followed by common letters differ significantly ($P < 0.05$)

4. Conclusion

A significant increase ($P < 0.05$) in the concentration of total FAA was registered during the drying and ripening period (23 days) of Šjenički sudžuk. The main FAA in the final product were glutamic acid (150 mg/100g dm), alanine (81.8 mg/100g dm), serine (81.6 mg/100g dm) and leucine (55.1 mg/100g dm). Along with gradual release of FAA, the formation and accumulation of BA was observed. Putrescine and tyramine were the predominant BA registered, indicating that on their release, the chief precursors, tyrosine and arginine, were used for metabolic activity by the aminogenic microbial population present. The registered histamine concentration (9.69 mg/kg) is much lower than recommended by EU regulations, which is considered a very positive finding of this research.

Acknowledgement

The authors would like to acknowledge the Serbian national project TR31032 financed by the Ministry of Education and Science of the Republic of Serbia.

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