TITLE: Effect of different ripening conditions on amino acids and biogenic amines evolution in Sjenički sudžuk

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Effect of different ripening conditions on amino acids and biogenic amines evolution in *Sjenički sudžuk*

Abstract

The aim of this study was to determine the influence of alternative ripening (thermo-hygrometric) conditions in the summer production season (batch S) on changes in free amino acids (FAA) and biogenic amines (BA) content in dry-fermented sausage *Sjenički sudžuk*, compared to traditional production during winter (batch W) in small/micro processing plant. Consequently to important proteolytic changes, both in batch W and especially in batch S, the concentration of total FAA increased ($P < 0.05$) over time, being 600 mg/100 g dm and 844 mg/100 g dm, respectively. By the end of ripening, the total concentration of six analyzed BA reached 399 mg/kg and even 2468 mg/kg in batches W and S, respectively. Predominant amines in batch W were putrescine and tyramine, and in batch S were cadaverine and putrescine. The concentration of these amines increased significantly ($P < 0.05$) throughout ripening within each of the observed periods of the year. Regarding histamine, the registered concentration was very low in samples of batch W (9.69 mg/kg), while the sausages of batch S were characterized by a much higher concentration of this harmful compound (333 mg/kg). In respect of this finding, the summertime production of *Sjenički sudžuk* in small/micro processing plants should be avoided until necessary manufacturing process modifications are implemented.

Keywords: Dry-fermented sausage; production season; thermo-hygrometric conditions; free amino acids; biogenic amines; food analysis; food composition.

1. Introduction

Sjenički sudžuk is dry-fermented sausage traditionally manufactured in a part of the Pešter plateau (approx. 1.000 m above sea level), a nearby area of the town of Sjenica, southwestern Serbia. Sjenica is considered the coldest place in Serbia, and one of the coldest towns in Europe, with an average annual temperature of 7.2°C (https://www.hidmet.gov.rs/). Nowadays, *Sjenički sudžuk* is produced according to an old recipe, using just beef, common salt and spices, without microbial starters, nitrites and other food additives (Ikonić et al., 2019). Traditionally, it is made during winter, when air temperatures are around 0°C or lower, and relative humidity is high. However, due to increased consumer demand, it is frequently produced outside of the winter season, even during the summertime, when climate conditions are less appropriate for this type of production. Therefore, the quality of "summer" sausages is usually lower, and certain food safety concerns may be raised, primarily related to microbial quality and the formation of biogenic amines (BA) (Roseiro et al., 2010; Ruiz-Capillas & Herrero, 2019; Schirone et al., 2022).

During the ripening of dry-fermented sausages the proteins undergo important degradation, resulting in the generation of various low molecular weight compounds, such as polypeptides, peptides, amino acids, aldehydes, organic acids, ammonia, amines, etc. (Dominguez et al., 2016; Hughes et al., 2002; Ikonić et al., 2013; Roseiro et al., 2010). This process is particularly pronounced at higher temperatures (≥ 25 °C) due to intensified activity of both endogenous muscle enzymes and those of microbial origin (Sentandreu, 2002; Toldrá, 2004). Hence, the concentration of free amino acids (FAA), the main precursors of BA, increases throughout processing. Consequently, when the conditions are favorable for the growth and metabolic activity of present decarboxylase-positive microbiota, it leads to the formation and accumulation of BA, the anti-nutritional nitrogenous bases which might represent toxicological effects on

human health (Dominguez et al., 2016; Eerola et al., 1998; Jairath et al., 2015; Kononiuk & Karwowska, 2020; Latorre-Moratalla et al., 2014; Rabie et al., 2014; Santos, 1996; Suzzi & Gardini, 2003). Besides meat products, BA can be found in different food commodities that contain proteins and/or free amino acids, such as fish and fish products, dairy products, fermented vegetables, olives, beer, wine, etc. (Dabadé et al., 2021; Đorđević et al., 2016; Ruiz-Capillas & Herrero, 2019; Santos, 1996). Thus, it is important to monitor the concentration of biogenic amines in fermented meat products and to control multiple factors which can contribute to their rise, such as quality of meat and other raw materials, manufacturing practices, processing stages and conditions, use of starter cultures, packaging solutions, storage and distribution conditions, etc. (Dabadé et al., 2021; Roselino et al., 2020; Ruiz-Capillas & Herrero, 2019; Schirone et al., 2022).

Current technical and scientific data about *Sjenički sudžuk* is still scarce, being important to improve the knowledge regarding physicochemical characteristics, microbial counts and proteolytic changes occurring during the smoking, drying and ripening process. Consequently, the aim of this research was to determine whether sausages alternatively processed during the summer production season (batch S), i.e. in different thermo-hygrometric conditions, have altered the evolution of FAA and BA when compared to corresponding products made in the winter period (W).

2. Material and methods

2.1 Standards and chemicals

The BA standards, i.e. tryptamine hydrochloride 99.0% (CAS No. 343-94-2), 2-phenylethylamine hydrochloride ≥98.0% (CAS No. 156-28-5), putrescine dihydrochloride ≥98.0% (TLC) (CAS No. 333-93-7), cadaverine dihydrochloride ≥99.0% (AT) (CAS No. 1476-39-7), histamine dihydrochloride ≥99.0% (AT) (CAS No. 56-92-8), tyramine hydrochloride ⩾98.0% (CAS No. 60-19-5), internal standard 1,7 – Diaminoheptane 98.0% (CAS No. 646-19-5) and dansyl chloride ≥99.0% (HPLC) (CAS Number: 605-65-29) were provided by Sigma–Aldrich (St. Louis, MO, USA). Trichloracetic acid (TCA) (CAS No. 76-03-9), sodium hydroxide p.a. (CAS No. 1310-73-2) and sodium hydrogen carbonate p.a. (CAS No. 144-55-8) were supplied by Lach-Ner (Neratovice, CZ). Acetone, HPLC grade (CAS No. 67-64-1) and perchloric acid 70% (CAS No. 7601-90-3) were acquired from Fisher Scientific (Loughborough, UK). Acetonitrile HPLC Gradient Grade (CAS No. 75-05-8), ninhydrin (CAS No. 485-47-2) and ammonium hydroxide p.a. (CAS No. 1336-21-6) were supplied by J.T. Baker-Avantor (Radnor Township, PA, USA), Biochrom (Cambridge, UK) and NRK Inženjering (Belgrade, RS), respectively.

2.2 Sausage preparation and samples

Samples of *Sjenički sudžuk* were manufactured according to traditional procedure in one micro/small 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 (pre-ripening). After that period, salted meat was ground through a 4 mm diameter mincing plate and mixed 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. The prepared batter was stuffed into natural casings with a diameter of approximately 40 mm and a length of approximately 50 cm. The ends of the sausage were tied off and bound together, forming a

horseshoe shape. Raw sausages were entirely processed in a traditional smoking/drying room for 23 days in winter (batch W) and summer production season (batch S). The smoking process lasted for the first 9 days in both seasons. The environmental (thermo-hygrometric) conditions during winter (W) and summer (S) production seasons in traditional practice 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. Physicochemical and microbial analyses were carried out on the day of sampling, and the rest of the sausages were homogenized, vacuum packed and stored at –20°C pending further analysis. Analyses for all samples were carried out in duplicate.

Fig. 1.

2.3 Physicochemical analyses

The pH of samples was determined using the portable pH meter Testo 205 (Testo SE & Co. KGaA, Titisee-Neustadt, DE) equipped with a combined penetration tip with the temperature probe. Water activity (a_w) was measured using LabSwift-aw measuring instrument (Novasina AG, Lachen, CH). Moisture content was quantified according to ISO 1442:1997, by heating the samples to 103°C ± 2°C until constant weight.

2.4 Microbial analysis

Total aerobic mesophilic bacteria (TAMB), lactic acid bacteria (LAB) and *Enterobacteriaceae* in sausage samples were enumerated according to ISO 4833-1:2013, ISO 15214:1998 and ISO 21528-2:2017, respectively. Mannitol Salt Agar (MSA) (Himedia Laboratories, Mumbai, IN) was used for enumerating *Micrococcaceae* (30 °C for 72 h). From each MSA plate, four colonies were further selected and characterized using Gram stain and

111 catalase reaction. In order to detect the presence of *Listeria monocytogenes* and *Salmonella spp*.
112 in 25 g samples, the recommended ISO standards were applied (ISO 11290-2:2017 and ISO
113 6579-1:2017, respectively). All experiments were performed in triplicate.

114 **2.5 Determination of free amino acids (FAA)**

115 Analyses of FAA in sausage samples were performed using ion exchange chromatography
116 with the utilization of Automatic Amino Acid Analyzer Biochrom 30+ (Biochrom, Cambridge,
117 UK), according to Rabie et al. (2014), with a few modifications. Briefly, 20 ml of 10 % (v/v)
118 trichloroacetic acid was added to 3 g of the sample, and the mixture was homogenized using T18
119 Basic Ultra Turrax (IKA-Werke GmbH & Co. KG, Staufen, DE), and the extract was filtered
120 through filter paper (FiltaTech, Fleury-les-Aubrais, FR). The extracts were centrifugated at 7000
121 × g for 15 min using a centrifuge 5804 R (Eppendorf, Hamburg, DE). The supernatant was
122 finally collected and filtered through 0.22 μm pore size PTFE filter (Plano, TX, USA), and
123 obtained filtrate was transferred to an HPLC vial (Agilent Technologies, Inc., Santa Clara, CA,
124 USA). FAA contents were determined by reaction with ninhydrin, with photometric detection at
125 2 wavelengths, 570 nm and 440 nm (for proline), and expressed as mg/100g of dry matter (dm).

126 **2.6 Determination of biogenic amines (BA)**

127 Six BA (tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine) were
128 determined according to the procedure described by Tasić et al. (2012). Briefly, around 2g of
129 each sample were weighed and put into a test tube with the appropriate amount of internal
130 standard and homogenized with 10 ml 0.4 M perchloric acid using T18 Basic Ultra Turrax (IKA-
131 Werke GmbH & Co. KG, Staufen, DE). After homogenization, samples were centrifuged for 10
132 min. at 900 × g, and the supernatant was decanted through filter paper into a 25 mL bottle.

133 Extraction was repeated by adding another 10 mL 0.4 M of perchloric acid solution to precipitate,
134 mixing using Vortex and centrifugation as before. Supernatants were merged and adjusted to 25
135 mL with 0.4 M perchloric acid. Afterward, 200 μL of 2 M NaOH was added into each sample
136 extract (1 mL) to make it alkaline and buffered with 300 μL of saturated $NaHCO_3$. Further, 2.0
137 mL of dansyl chloride solution was added and the resulting mixture was incubated at 40 °C for
138 45 min. With the addition of 100 μL ammonia, residual dansyl chloride was removed. 30 min
139 later mixture was adjusted to 5.0 mL with acetonitrile, filtered (0.45 μm, PTFE, Plano, TX, USA)
140 and subjected to analysis. BA were determined as their dansyl derivatives, using liquid
141 chromatography (Agilent 1200 series, Agilent Technologies, Inc, Santa Clara, CA, USA),
142 equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies, Inc.,
143 Santa Clara, CA, USA), a binary pump, an online vacuum degasser, an autosampler and a
144 thermostated column compartment, on Eclipse XDB-C18, 1.8 μm, 4.6 x 50 mm column (Agilent
145 Technologies, Inc., Santa Clara, CA, USA). The solvent gradient was performed by varying the
146 proportion of solvent A (acetonitrile) to solvent B (water) as follows: initial 50% B; linear
147 gradient to 10% B in 7.6 min,10% B to 10 min; linear gradient to 50% B in 2 min. The system
148 was equilibrated 3 min. before the next analysis. The flow rate was 1.5 mL/min, the column
149 temperature was 40°C, 5 μL of the sample was injected. The spectra were acquired in the range
150 of 190-400 nm, and separated amines were detected at a wavelength of 254 nm. BA contents are
151 expressed as mg/kg of sample.

152 **2.7 Statistical analyses**

153 Two-way ANOVA (Statistica 13.3, TIBCO Software Inc., Palo Alto, CA, USA) was used to
154 analyse the effects of processing conditions and ripening time on the observed variables, and
155 post-hoc (Duncan) test was performed for comparison of mean values. Differences were

considered significant at $P < 0.05$. Principal component analysis (PCA) was performed to evaluate and classify the main variables of all samples using the same software package.

3. Results and discussion

3.1 pH and water activity (a_w)

Mean values of pH and a_w of sausages produced under different thermo-hygrometric conditions are shown in Fig. 2. Both analyzed values were greatly influenced by the air temperature in the traditional smoking/drying room, being on average 6.5°C higher for batch S. The difference in thermo-hygrometric conditions between batches W and S was particularly pronounced during the smoking phase, when the average temperatures were 12.1°C and 28.5°C, respectively (Fig. 1). Consequently, development of the natural microflora was affected, resulting in the faster fermentation process and more intensive pH decline in sausages of batch S. After 7 days of fermentation the pH had fallen from an initial value of 5.65 to 5.21 in sausages of batch W and from 5.86 to 5.26 in products of batch S. Thus, a larger pH drop was registered in sausages of batch S compared to those of batch W, amounting 0.6 and 0.45 units, respectively. Afterward, pH remained constant in sausages of batch W ($P > 0.05$) or started a gradual increase in samples of batch S ($P < 0.05$), due to proteolytic reactions, i.e. liberation of peptides, amino acids and other non-protein nitrogenous compounds (Ikonić et al., 2013; 2016; Rocchetti et al., 2021; Spaziani et al., 2009). After 23 days of drying and ripening, the weight loss was about 42.7% and 43.6% (data not shown) and, as a consequence, a_w gradually and significantly ($P < 0.05$) decreased to 0.84 and 0.77 in sausages of batches W and S, respectively. Thus, the obtained results indicated a more intensive drying process of batch S.

178 Fig. 2.

179 **3.2 Microbial counts**

180 The microbial counts (Log CFU/g) at different ripening stages of sausages from batches W
181 and S are depicted in Fig. 3. Overall, significant differences ($P < 0.05$) were observed between
182 tested product batches for all analyzed microbial groups, except for *Micrococcaceae* counts after
183 7 and 15 days of ripening. As it was previously mentioned, the higher environmental temperature
184 used for batch S accelerated the fermentation process, increasing TAMB and LAB counts up to
185 8.08 log CFU/g after only 3 days of drying and ripening, while in their counterparts from batch
186 W, those microorganisms reached the highest level (7.99 log CFU/g and 7.62 log CFU/g,
187 respectively) at day 15. Regarding the *Micrococcaceae* counts, the highest values were registered
188 at the beginning of the ripening process (0 day), being 5.16 and 6.00 log CFU/g in samples of
189 batch W and S, respectively. Until the end of the ripening period (23rd day), significant reduction
190 ($P < 0.05$) of MSA agar counts was observed in batches W and S, amounting approx. 1.5 and 3.0
191 log units, respectively. In both batches, *Micrococcaceae* counts seemed to be greatly affected by
192 intensive growth and activity of LAB, i.e. rapid acidification, confirming previously published
193 findings of a number of authors concerning the poor competing ability of *Micrococcaceae* against
194 active acidogenic bacteria (LAB) (Hughes et al., 2002; Ikonić et al., 2016; Rocchetti et al., 2021;
195 Spaziani et al., 2009). With regard to *Enterobacteriaceae* counts, initial numbers were quite high,
196 amounting 4.90 and 6.66 log CFU/g in batches W and S, respectively. It could indicate the poor
197 hygienic quality of the raw materials and inappropriate processing conditions. Note that salted
198 beef underwent 7 days long pre-ripening phase before sausage processing. Similar levels of
199 *Enterobacteriaceae* in beef after 6 days of chilled storage were reported by Triki et al. (2018).
200 However, a significant decrease ($P < 0.05$) in *Enterobacteriaceae* counts was registered during

the drying and ripening period, especially in products of batch S, where these microbes were not registered after only three days. The reduction in *Enterobacteriaceae* number could be explained by the growth of LAB associated with pH decline (Barbieri et al., 2019; Kononiuk & Karwowska, 2020; Lorenzo et al., 2014; Rocchetti et al., 2021; Sun et al., 2018). Additionally, the pathogenic bacteria *Salmonella spp.* was not detected in 25 g of any sample of sausages from both batches during the whole ripening period, while *L. monocytogenes* was registered in the first 7 days in sausages of batch W, but it disappeared afterward (data not shown).

Fig. 3.

3.3 Free amino acids (FAA)

The evolution in the concentration of FAA during processing within two production seasons is represented in Table 1. The total FAA concentration in the raw sausage batter of batch W was 388 mg/100g dm, while this value for batch S was slightlyhigher, amounting 472 mg/100g dm. Throughout 23 days of drying and ripening in different thermo-hygrometric conditions (batches W and S), an increase in the concentration of most amino acids was registered, giving a significant rise ($P < 0.05$) to a final concentration of total FAA, being 600 mg/100g dm and 884 mg/100g dm, respectively. The amino acids which primarily contributed to this increase in batch W were glutamic acid, leucine and valine, followed by threonine, lysine and phenylalanine. Regarding batch S, the FAA most responsible for the registered increase in total concentration were leucine, valine, isoleucine, threonine, methionine and glycine. Gradual release of amino acids during ripening is characteristic of dry-fermented sausages. The registered increasing trend in *Sjenički sudžuk* is in accordance with previously reported findings by a number of authors (Dominguez et al., 2016; Hughes et al., 2002; Latorre-Moratalla et al., 2014; Rabie et al., 2014).

On the contrary, the concentration of serine, tryptophan and arginine decreased significantly ($P < 0.05$) in batch W, while it was the case with glutamic acid in batch S. This reduction may indicate more intense uptake by bacteria and potential conversion to BA, comparing to their formation during ripening (Hughes et al., 2002; Rabie et al., 2014).

The predominant FAA in *Sjenički sudžuk* from batch W were glutamic acid (60.8-150 mg/100g dm), alanine (69.8-81.8 mg/100g dm) and serine (109-81.6 mg/100g dm). In sum, they accounted for about 62% and 52% of the total FAA in raw vs. ripened sausage, respectively. These findings confirm previous reports regarding the highest prevalence of glutamic acid (Lorenzo & Franco, 2012) and alanine (Aro Aro et al., 2010) in fermented sausages. In respect to products of batch S, the main amino acids registered in raw sausage were glutamic acid (158 mg/100g dm), alanine (81.1 mg/100g dm) and glycine (31.7 mg/100g dm), accounting for about 60% of total FAA. After 23 days of ripening, leucine (133 mg/100g dm), alanine (111 mg/100g dm) and valine (94.8 mg/100g dm) were the most represented FAA, accounting for about 40% of total FAA concentration. These results are in partial accordance with those obtained by Rabie et al. (2014), 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. (2016), who observed leucine, cysteine and phenylalanine as chief FAA in non-started dry-fermented foal sausage.

Table 1.

3.4 Biogenic amines (BA)

Tyrosine, histidine, arginine, lysine and phenylalanine are the main precursors of dietary BA, tyramine, histamine, putrescine, cadaverine and phenylethylamine, respectively (Latorre-Moratalla et al., 2014; Rabie et al., 2014). Due to proteolytic changes, most of them underwent

significant increase ($P < 0.05$) during the drying and ripening of *Sjenički sudžuk,* both in the winter and summer production seasons. FAA availability, in combination with the activity of decarboxylase-positive microbiota, usually enables the formation and accumulation of BA in fermented sausages (Ikonić et al., 2021; Jairath et al., 2015; Latorre-Moratalla et al., 2014; Rabie et al., 2014; Roseiro et al., 2010; Roselino et al., 2020). This fact also appears to hold in the case of *Sjenički sudžuk,* regardless of the production season.

Changes in the concentration of BA throughout ripening in winter (W) and summer (S) production seasons are depicted in Table 2. The concentration of total BA in batch W ranged from 0 to 399 mg/kg. The relatively low level of total BA detected in sausages from batch W along the processing period was most likely the consequence of unfavorable conditions for the growth and activity of aminogenic microbiota, i.e. rather low temperature during the winter production period, being on average 9.52°C (Fig. 1(W)) (Ikonić et al., 2013; Roseiro et al., 2010).

On the contrary, the total BA content in products of batch S was much higher, ranging from 242 to as much as 2468 mg/kg, thus reflecting the effect of favourable smoking and overall processing temperature (avg. 28.5°C and avg. 15.9°C, respectively (Fig. 1(S)) on distinct evolution of microbial populations. Obviously, the concentration of total BA in batch S was 2.5 times higher than the maximum threshold of 1000 mg/kg, which has been considered dangerous for human health (Ikonić et al., 2013; Kononiuk & Karwowska, 2020; Li et al., 2019; Rabie et al., 2014; Santos, 1996).

Putrescine was the most abundant amine found in *Sjenički sudžuk* after 23 days of processing in the winter period (batch W) and the second most common amine registered in sausages of batch S. Its concentration increased significantly ($P < 0.05$) as ripening time elapsed, ranging from 0 to 212 mg/kg in batch W and from 41.8 to 570 mg/kg in batch S. Putrescine level found in samples of batch W is in accordance with previous reports regarding this amine in dry-fermented

beef sausage (Rabie et al., 2014) and Portuguese traditional dry-fermented sausage alternatively processed in the chilling room (Roseiro et al., 2010). Conversely, it is higher than those determined in non-started sausages by Latorre-Moratalla et al. (2014) and Domínguez et al. (2016) and lower than the value obtained by Roseiro et al. (2010) in Portuguese sausage entirely processed in traditional smoking/drying room, as well as the value reported by Van Ba et al. (2016) for pork fermented sausage produced without starter culture inoculation. The concentration of putrescine 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* (Ikonić et al., 2021; Jairath et al., 2015; Ren et al., 2022; Roseiro et al., 2010; Sun et al., 2018). However, the putrescine concentration continued to rise in sausages of batch S throughout the ripening period, even though these microbes have not been registered on the 3rd day of ripening and further on. Hence, the previous assumption regarding the *Enterobacteriaceae* capability to release decarboxylases that remain active for extended periods (Bover-Cid et al., 2001; Roseiro et al., 2010) seems to be confirmed in this study.

Tyramine was the second and the third most common amine found in *Sjenički sudžuk* after 23 days of drying and ripening of batches W (147 mg/kg) and S (388 mg/kg), respectively, endorsing previously reported findings regarding its high abundance in dry-fermented sausages. Tyramine concentration is closely related to the presence and metabolic activity of LAB due to their potential in tyrosine decarboxylation (Barbieri et al., 2019; Ikonić et al., 2021; Jairath et al., 2015; Roseiro et al., 2010; Suzzi & Gardini, 2003; Van Ba et al., 2016). This amine is directly influenced by the level of tyrosine, which remained essentially constant in batch W or even decreased in batch S, indicating that the release of this amino acid was used for metabolic reactions of present microbiota and the formation of tyramine (Latorre-Moratalla et al., 2014; Rabie et al., 2014). The levels of tyramine found in this work are consistent with those

encountered before in several European dried sausages (Dabadé et al., 2021; Domínguez et al., 2016; Ikonić et al., 2021; Latorre-Moratalla et al., 2014; Roseiro et al., 2010; Suzzi & Gardini, 2003).

With respect to histamine, the most important BA from the toxicological and hygienic aspect, slight accumulation was registered after 23 days in batch W, while the extensive increase was found in batch S, amounting 9.69 mg/kg and 333 mg/kg, respectively. Thus, the concentration of histamine in *Sjenički sudžuk* of batch W was much lower than its allowable limit in food (100 mg/kg) (Dominguez et al., 2016; Jairath et al., 2015; Rabie et al., 2014), and much lower than the level reported by EFSA (2011) for European fermented sausages (approx. 25 mg/kg). On the contrary, the content of this harmful compound was very high in sausages of batch S, even higher than the value reported by Rabie et al. (2014) in fermented turkey sausage after 28 days of storage (263 mg/kg dm) and by Li et al. (2019) in traditional Chinese sausage from Baoding (209.6 mg/kg). However, the concentration of histamine in samples of batch S was lower than the value registered in Egyptian fermented beef sausages after 1 month of storage (768 mg/kg dm) (Rabie et al., 2010). The aforementioned levels of histamine may pose a risk to public health, i.e. concentration higher than 100 mg/kg may cause poisoning (Dominguez et al., 2016; Eerola et al., 1998; Jairath et al., 2015; Rabie et al., 2010, 2014).

Moreover, the sum of vasoactive amines (histamine, tyramine, tryptamine, phenylethylamine) did not exceed 200 mg/kg in sausages of batch W. Conversely, the total amount of these four BA in samples of batch S was much higher than the maximum threshold, indicating misapplication of good manufacturing practice during the processing of *Sjenički sudžuk* in summer production season (Eerola et al., 1998).

Previously shown results regarding the BA concentration in sausages of batch S and many of recent publications analyzing the effect of starter culture inoculation on the reduction of biogenic

amines accumulation in fermented sausages (Dominguez et al., 2016; Kononiuk & Karwowska, 2020; Ren et al., 2022; Rocchetti et al., 2021; Van Ba et al. 2016) suggest that further production of *Sjenički sudžuk* outside the winter production season should be conducted with the addition of appropriate starter culture.

Table 2.

3.5 Principal component analysis (PCA)

PCA was performed to obtain linear combinations of BA and their FAA precursors, microbial counts and physiochemical properties of *Sjenički sudžuk* throughout ripening in winter (W) and summer (S) production seasons. As reported in Table 3 and plotted in Fig. 4, the results of PCA revealed that the first three principal components accounted for 93.80% of the total variance of data. PC1 mainly comprised the positive effect of BA, allowing clear separation of the batches produced in different seasons (W vs. S). Thus, samples of batch S, except S0, were allocated at the positive side of PC1, close to all BA and most of the FAA precursors. On the contrary, the negative side of PC1 generated all samples of batch W, indicating low levels of BA and being highly influenced by a_w and *Enterobacteriaceae* counts. PC2 showed a clear difference between sample S0 and all other samples, as it obtained higher scores for pH and *Micrococcaceae* counts. Finally, PC3, mainly associated with TAMB and LAB counts, separated the samples from batch W into two groups, i.e. W0 and W3 vs. W7, W15 and W23. Also, according to the same microbial indicators, samples S0 and S3 were allocated at the positive side of PC3, indicating very high initial numbers of TAMB and LAB in these samples of batch S.

Fig. 4 and Table 3.

4. Conclusion

Thermo-hygrometric conditions applied during the smoking, drying and ripening period in the summer production season (batch S) resulted in a more intensive release of FAA, as well as formation and accumulation of BA, compared to batch W. Moreover, the concentrations of histamine and total BA in batch S were much higher than themaximum thresholds (100 mg/kg and 1000 mg/kg, respectively), which are considered dangerous for human health, indicating inappropriate elaboration of sausages from this batch, such as incorrect manufacturing practices and conditions. Thus, the summertime production of *Sjenički sudžuk* in small/micro processing plants should be avoided until necessary manufacturing process modifications are implemented, that will ensure less formation and accumulation of BA, i.e. appropriate control and regulation of thermo-hygrometric conditions, use of decarboxylase negative starter cultures, etc.

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CRediT authorship contribution statement

Predrag Ikonić: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Data curation, Visualization. **Marija Jokanović**: Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing. **Nedim Ćućević**: Conceptualization, Formal analysis, Funding acquisition, Resources. **Tatjana Peulić**: Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision. **Ljubiša Šarić**: Methodology, Formal analysis, Investigation, Writing - review & editing. **Zorica**

Tomičić: Formal analysis, Investigation. **Snežana Škaljac:** Formal analysis, Investigation, Resources, Project administration. **Jovana Delić:** Formal analysis, Investigation. **Brankica Lakićević:** Methodology, Validation, Resources. **Igor Tomašević:** Methodology, Validation, Resources.

Declarations of interest: none

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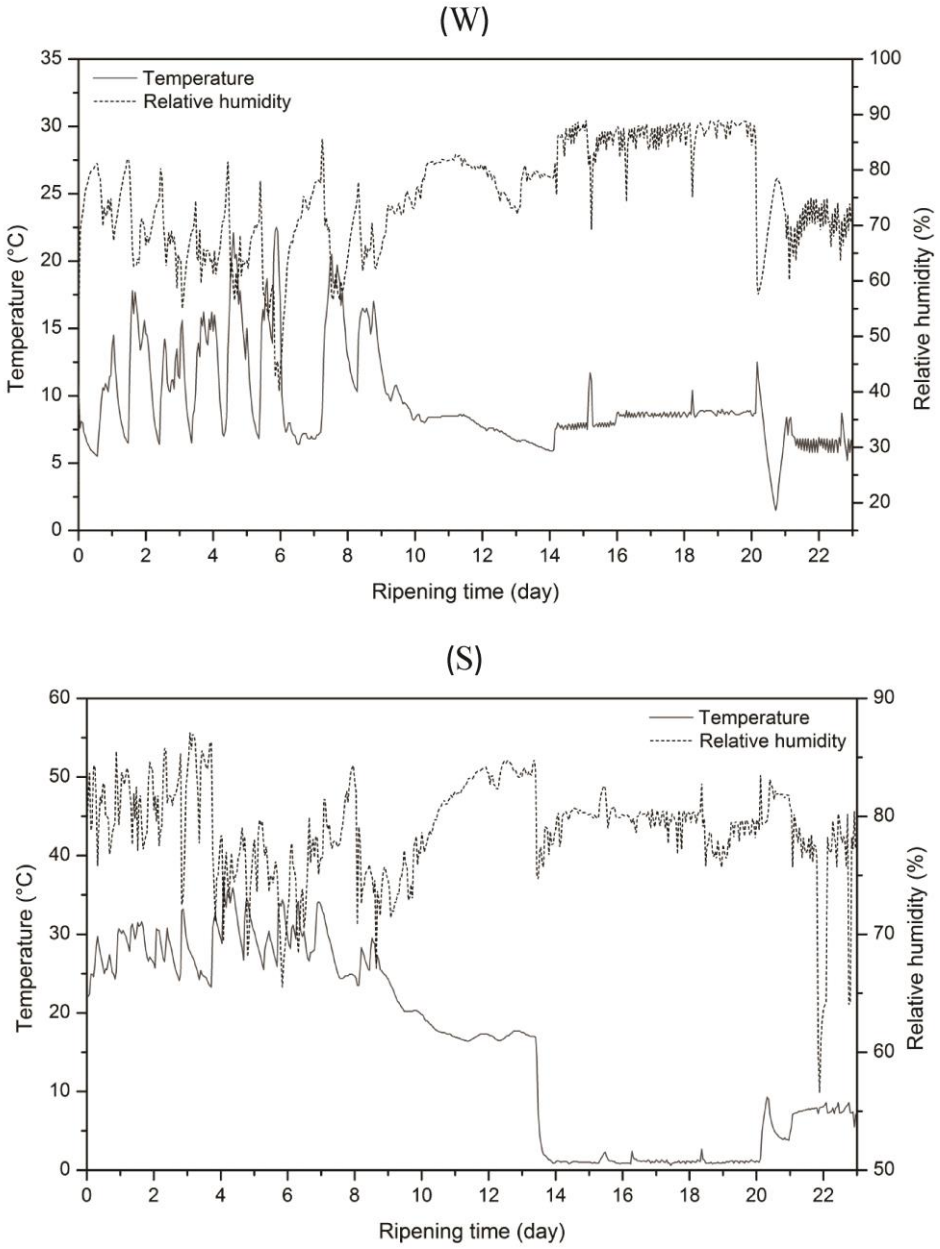

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2 Fig. 1. Environmental temperature (°C) and relative humidity (%) recorded throughout
3 processing of Sjenički sudžuk: winter production season (W), summer production season (S).

4

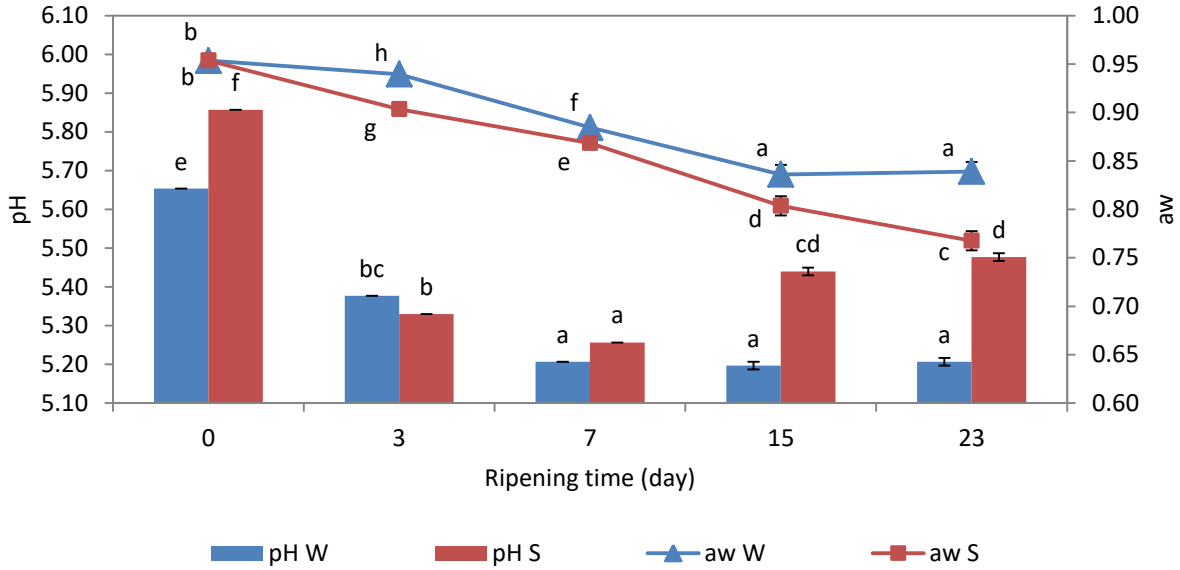

Fig. 2. Changes in pH and water activity (a_w) of *Sjenički sudžuk* throughout ripening in winter (W) and summer (S) production season (mean ± standard deviation). Mean values for each physicochemical indicator not marked by common letter differ significantly ($P < 0.05$)

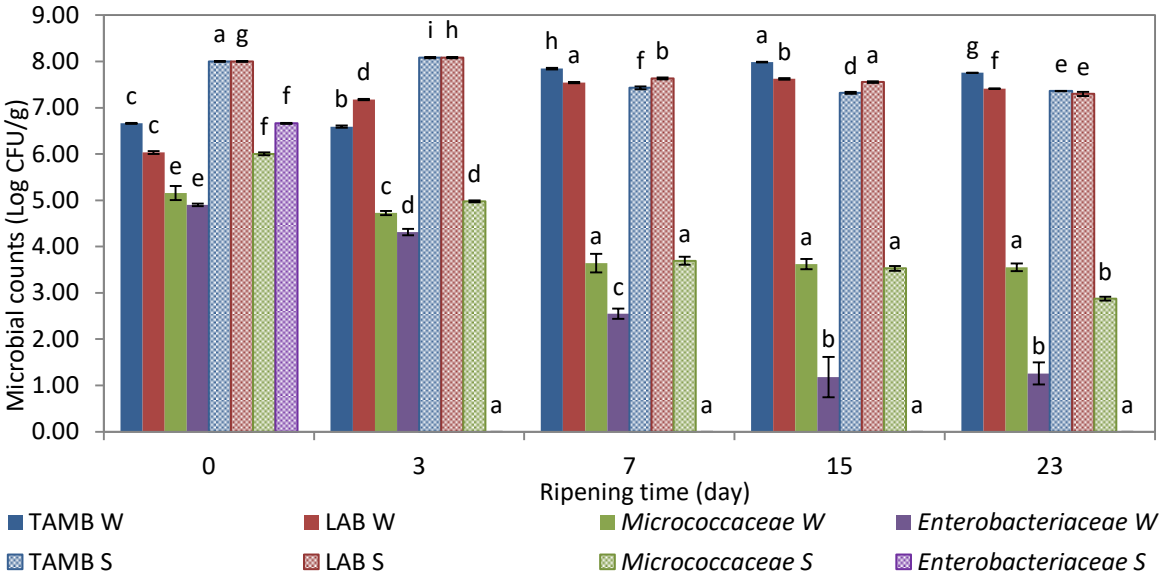

Fig. 3. Changes in microbial counts (Log CFU/g) in *Sjenički sudžuk* throughout ripening in winter (W) and summer (S) production season (mean ± standard deviation). Mean values for each microbial group not marked by common letter differ significantly ($P < 0.05$). TAMB - total aerobic mesophilic bacteria; LAB – lactic acid bacteria.

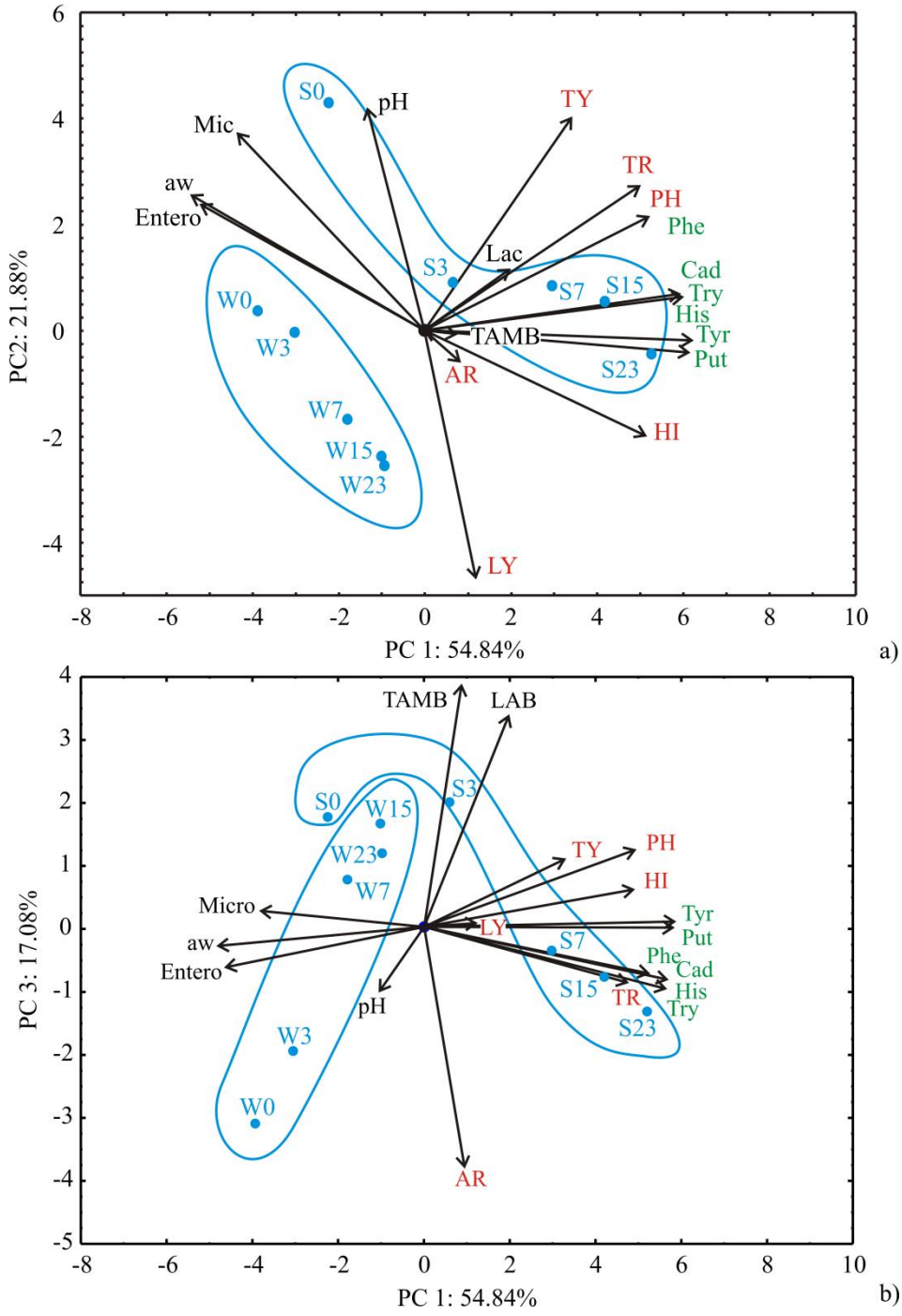

Fig. 4. Principal Component Analysis (PCA) for physicochemical properties, microbial counts, FAA precursors of BA and BA of *Sjenički sudžuk* throughout ripening in winter (W) and summer (S) production season. a) Projection of variables and samples in plane defined by PC1 and PC2. b) Projection in plane defined by PC1 and PC3. TAMB - total aerobic mesophilic bacteria; LAB – lactic acid bacteria; Micro – *Micrococcaceae;* Entero – *Enterobacteriaceae;* TY – tyrosine; HI – histidine; AR – arginine; LY – lysine; PH – phenylalanine; Tyr – tyramine; His – histamine; Put – putrescine; Cad – cadaverine; Phe – phenylethylamine.

Table 1. Changes in concentration (mg/100g dm) of free amino acids in *Sjenički sudžuk* throughout ripening in winter (W) and summer (S) production season (mean ± standard deviation).

Processing time (day)	0		3		7		15		23		PT	B	PT x B
Batch	W	S	W	S	W	S	W	S	W	S			
Aspartic acid	7.27 ± 0.6a	6.50 ± 0.5a	6.85 ± 0.3a	14.8 ± 1.1b	9.94 ± 1.5c	21.1 ± 1.2d	13.8 ± 2.2b	25.9 ± 0.8e	15.0 ± 3.7b	33.1 ± 0.5f	*	*	*
Threonine	7.54 ± 0.6c	23.6 ± 0.6ab	12.6 ± 3.0d	30.2 ± 1.1e	19.2 ± 6.5a	51.4 ± 1.6f	23.5 ± 0.8ab	61.5 ± 1.5g	25.4 ± 1.6b	68.0 ± 1.4h	*	*	*
Serine	109 ± 13g	10.1 ± 2.3a	104 ± 3.6fg	17.0 ± 1.5ab	96.5 ± 6.5f	24.3 ± 1.7bc	83.7 ± 1.4e	27.1 ± 1.5cd	81.6 ± 7.9e	35.6 ± 1.8d	ns	*	*
Glutamic acid	60.8 ± 5.0b	158 ± 3.0d	86.9 ± 6.6e	51.8 ± 1.2ab	109 ± 6.4f	40.5 ± 11a	141 ± 8.7c	41.3 ± 3.1a	150 ± 8.1cd	43.6 ± 1.6a	*	*	*
Proline	3.92 ± 0.3bc	5.59 ± 1.7b	11.2 ± 0.4a	15.9 ± 1.3c	11.1 ± 0.6a	3.31 ± 2.3b	12.5 ± 1.1a	18.1 ± 2.1c	12.4 ± 0.2a	17.3 ± 1.7c	*	*	*
Glycine	11.3 ± 0.3b	31.7 ± 0.5d	12.8 ± 1.4b	44.2 ± 1.2e	15.9 ± 1.5a	68.9 ± 2.0c	17.8 ± 1.4a	74.5 ± 3.4f	18.9 ± 2.8a	71.3 ± 0.8c	*	*	*
Alanine	69.8 ± 6.0a	81.1 ± 3.3a	75.3 ± 10.3a	107 ± 3.8b	79.5 ± 7.5a	128 ± 5.1c	82.4 ± 9.1a	119 ± 6.1bc	81.8 ± 3.9a	111 ± 0.3b	*	*	*
Cysteine	16.9 ± 0.6a	11.4 ± 2.1c	18.4 ± 3.3ab	10.0 ± 2.1c	20.2 ± 2.4ab	21.1 ± 2.8ab	21.7 ± 4.1ab	22.6 ± 3.1ab	22.9 ± 4.0b	21.3 ± 3.8ab	*	*	ns
Valine	12.7 ± 2.4b	30.8 ± 2.0a	17.0 ± 1.4b	54.4 ± 2.3f	25.5 ± 2.0e	90.6 ± 1.6c	32.1 ± 2.5a	97.3 ± 2.8d	34.7 ± 4.1a	94.8 ± 3.1cd	*	*	*
Methionine	9.12 ± 1.0ab	8.14 ± 1.1a	10.2 ± 1.7ab	28.2 ± 2.1e	13.4 ± 2.0bc	48.1 ± 6.1d	15.9 ± 4.5c	52.5 ± 2.3d	16.4 ± 1.2c	48.0 ± 2.1d	*	*	*
Isoleucine	11.9 ± 1.0b	22.1 ± 0.9a	11.5 ± 0.9b	38.7 ± 2.0e	16.6 ± 2.9c	68.1 ± 2.1d	20.1 ± 4.2ac	73.7 ± 1.2f	21.3 ± 0.8a	69.2 ± 4.3d	*	*	*
Leucine	16.8 ± 0.5a	8.43 ± 2.5a	25.6 ± 5.6e	85.6 ± 4.5g	41.4 ± 7.4f	137 ± 9.7cd	51.6 ± 3.7b	143 ± 2.8d	55.1 ± 4.4b	133 ± 3.1c	*	*	*
Tyrosine	1.33 ± 0.5a	9.85 ± 1.6c	1.57 ± 0.6a	7.19 ± 2.4b	1.73 ± 0.8a	7.10 ± 1.0b	1.61 ± 0.6a	6.95 ± 1.2b	1.61 ± 0.4a	6.88 ± 1.1b	ns	*	ns
Phenylalanine	3.43 ± 0.9c	31.4 ± 4.3a	6.52 ± 1.2c	26.5 ± 3.8f	12.6 ± 1.8d	34.8 ± 1.3ab	16.4 ± 3.0de	37.2 ± 2.7b	17.5 ± 2.6e	34.1 ± 2.6ab	*	*	*
Histidine	5.61 ± 0.3d	4.99 ± 0.9d	6.49 ± 0.7d	13.2 ± 1.6c	9.40 ± 1.0a	10.1 ± 1.0ab	10.8 ± 2.8abc	12.4 ± 0.4bc	11.1 ± 0.9abc	17.2 ± 2.5e	*	*	*
Tryptophan	10.7 ± 0.5c	25.1 ± 5.0e	8.49 ± 0.6bc	18.8 ± 2.1e	3.81 ± 0.2abc	43.0 ± 6.2d	1.92 ± 0.1ab	40.9 ±7.7d	0.50 ± 0.2a	40.0 ± 4.7d	*	*	*

Lysine	14.6 ± 1.1bc	2.92 ± 0.3h	13.1 ± 0.9ab	9.29 ± 0.9a	22.0 ± 2.9de	12.2 ± 1.3ab	28.0 ± 3.8fg	18.1 ± 2.5cd	30.6 ± 2.6g	26.0 ± 4.4ef	*	*	*
Arginine	15.0 ± 2.9b	0.69 ± 0.3c	15.0 ± 2.0b	3.42 ± 0.5a	8.03 ± 0.8d	10.5 ± 0.7de	3.07 ± 0.5ac	10.9 ± 0.8e	3.94 ± 0.4a	14.0 ± 2.8b	ns	*	*
Total FAA	388 ± 11d	472 ± 33ef	443 ± 5.5de	576 ± 36ab	516 ± 41a	820 ± 59c	578 ± 46ab	883 ± 46c	600 ± 1.2b	884 ± 43c	*	*	*

[a-d] Mean values within the same row not followed by common letter differ significantly ($P < 0.05$)

PT – processing time; B – batch; FAA – free amino acids

ns = not significant.

* $P < 0.05$

Table 2. Changes in concentration (mg/kg) of biogenic amines in *Sjenički sudžuk* throughout ripening in winter (W) and summer (S) production season (mean ± standard deviation).

Processing time (day)	0		3		7		15		23		PT	B	PT x B
Batch	W	S	W	S	W	S	W	S	W	S			
Tryptamine	NDa	NDa	NDa	31.5 ± 1.8b	NDa	85.1 ± 2.1c	NDa	105 ± 1.6d	NDa	108 ± 1.7e	*	*	*
Phenylethylamine	NDa	62.1 ± 1.7c	36.0 ± 1.3b	71.8 ± 2.3d	NDa	119 ± 3.6e	NDa	146 ± 5.1f	NDa	167 ± 8.1g	*	*	*
Putrescine	NDa	41.8 ± 2.3b	NDa	271 ± 9.3f	91.9 ± 6.2c	426 ± 7.8g	190 ± 1.0d	515 ± 12h	212 ± 10e	570 ± 8.7i	*	*	*
Cadaverine	NDb	56.9 ± 4.4c	NDb	301 ± 9.8d	20.4 ± 2.1a	632 ± 12e	25.3 ± 0.7a	815 ± 15f	30.8 ± 3.8a	901 ± 19g	*	*	*
Histamine	NDa	NDa	NDa	101 ± 6.4b	NDa	241 ± 7.8c	NDa	301 ± 16d	9.69 ± 0.7a	333 ± 13e	*	*	*
Tyramine	NDb	81.2 ± 3.6d	48.5 ± 2.9c	183 ± 6.7f	102.8 ± 3.6e	295 ± 8.7g	138 ± 3.6a	341 ± 11h	147 ± 8.3a	388 ± 23i	*	*	*
Total BA	NDb	242 ± 3.2a	84.4 ± 4.2c	960 ± 36f	215 ± 12a	1798 ± 35g	353 ± 5.3d	2222 ± 37h	399 ± 23e	2468 ± 28i	*	*	*

[a-d] Mean values within the same row not followed by common letter differ significantly ($P < 0.05$)

PT – processing time; B – batch; BA – biogenic amines

ND – not detected

* $P < 0.05$

Table 3. Contribution of each variable (%) to first three principal components.

Variable	PC 1 (54.84%)	PC 2 (21.88%)	PC 3 (17.08%)
Tryptamine	9.25	0.46	2.03
Phenylethylamine	8.10	3.76	1.07
Putrescine	9.96	0.16	0.00
Cadaverine	9.44	0.55	1.43
Histamine	9.37	0.37	1.92
Tyramine	10.09	0.03	0.01
Tryptophan	6.59	7.04	1.55
Phenylalanine	7.14	4.47	3.00
Arginine	0.26	0.30	29.66
Lysine	0.44	21.09	0.01
Histidine	7.01	3.69	0.73
Tyrosine	3.19	15.37	2.38
TAMB	0.22	0.00	29.97
LAB	1.17	1.25	22.94
Micrococcaceae	4.29	13.10	0.14
Enterobacteriaceae	6.34	5.46	0.86
pH	0.33	16.80	2.10
a_w	6.81	6.10	0.18

TAMB - total aerobic mesophilic bacteria; LAB – lactic acid bacteria