Hindawi
Journal of Food Quality
Volume 2022, Article ID 2480746, 12 pages
https://doi.org/10.1155/2022/2480746

Research Article

Digital Evaluation of Nitrite-Reduced "Kulen" Fermented Sausage Quality

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Received 19 November 2021; Revised 28 February 2022; Accepted 29 April 2022; Published 6 June 2022

Academic Editor: Ayon Tarafdar

This study aimed to evaluate nitrite reduction impact on geometry, colour, chemical, microbiological, and sensory traits of dry sausage (*kulen*) traditionally prepared with red hot paprika powder. Three batches of *kulen* with different nitrite levels were produced and assessed: N110 (control with 110 mg/kg of sodium nitrite), N55 (55 mg/kg of sodium nitrite), and NF (without sodium nitrite). Samples for the analyses were taken on production day, after 8, 16, 24, 32, and 40 days of ripening and after 50 and 100 days of storage. Four novel digital methods for quality assessment were deployed such as computer vision system (CVS), three-dimensional (3D) laser imaging, oral processing, and temporal dominance of sensations (TDS). Reduction and removal of nitrites from the formulation of *kulen* did not result in significant ($P < 0.01$) differences in lightness (L^*), redness (a^*), and yellowness (b^*) of the sausage surface, meat, and fat parts that were measured independently by means CVS. Sausages produced by 50% nitrite reduction (N55) showed no significant ($P < 0.01$) differences in terms of geometrical, chemical, colour, microbiological, and oral processing parameters compared with the control (N110) batch. On the other hand, the complete removal of nitrites from *kulen* formulation negatively affected biogenic amine levels and oral processing properties of the product. Nitrite reduction showed no significant effect on TDS curves among the batches. The results of this study indicate that nitrite content in traditional *kulen* can be reduced by 50% (55 mg/kg of sodium nitrite) without adversely affecting the various quality properties of the product.

1. Introduction

Nitrites are food additives widely used in meat curing due to their positive effects on oxidative stability, flavour enhancement, and antimicrobial effect on pathogen microorganisms especially *Clostridium botulinum* [1–3]. However, the most important effect of nitrites in processed meat is the development of characteristic bright red colour as a result of nitrosylmyoglobin ($MbFe^{II}NO$) formation [4]. On the other hand, the use of nitrites is commonly associated with negative health effects as a result of their interaction with secondary amines when carcinogenic N-nitrosamines may be formed [5]. It is believed that the formation of these compounds could be one of the reasons why the

International Agency for Research on Cancer (IARC) classified processed meat as carcinogenic to humans (Group 1) [6, 7]. Besides their carcinogenic effects, nitrosamines were found to be mutagenic and may induce cardiovascular diseases [8].

In recent years, as a result of consumers becoming more aware of the potential health risks of food additives, demand for "clear label" food is constantly increasing [9]. This is why the corporate social responsibility activities of meat companies became more focussed on nutrition-based initiatives [10]. To meet consumer demands, it is necessary to either abandon the use of additives or reduce their content or replace them, provided that safety and quality are not compromised. Recently, certain vegetables (beetroot, celery, lettuce, spinach, and radishes) that are high in nitrates have been proposed as green alternatives to nitrates and nitrites in processed meat [4, 11]. Although nitrates are coming from natural sources, they are still considered as compounds that are potentially hazardous to human health, as they can convert into nitrites and form nitrosamines [11]. Moreover, certain spices (rosemary, mace, oregano, and red paprika) with strong antibacterial and antioxidant activity have also been proposed as good nitrite alternatives [11–13]. However, as these spices are rich in volatile compounds, the main problem in the application of high quantities of these spices is the deterioration of the sensory properties of the product.

Kulen is a traditional fermented sausage produced in the Balkan region and it is characterised by the use of large quantities of red hot paprika powder in its formulation (up to 3%) [14]. Red paprika contains carotenoids, including red pigments like capsanthin, capsorubin, and cryptoxanthin, which are the main compounds responsible for the red colour of the sausage [15, 16]. Besides their effect on colour, carotenoids are compounds with powerful antioxidant activity that is considered as an important attribute when it comes to processed meat [17]. In addition, many studies reported antimicrobial activity of *Capsicum* spp. on some pathogen bacteria including *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Salmonella enterica*, *Clostridium sporogenes*, and *Clostridium tetani* [18, 19]. Some *Capsicum* spp. contain up to 476 mg/kg of nitrates some of which may be reduced to nitrites during the ripening of fermented sausages by the activity of the microbial population [3, 20]. Additionally, paprika is capable of modifying food flavour as it contains many volatile compounds [21]. Hence, Tang et al. [13] proposed the application of red chili pepper as a nitrite alternative for the improvement of antioxidant, microbial, textural, and sensory traits, and reduction of many potentially harmful microbes in dry sausages.

Most of the previous papers on nitrite reduction in dry sausages were done with model systems that traditionally do not involve the use of red paprika powder in the formulation. Since red paprika was found to have positive effects on the control of pathogens and it is rich in flavour and colour compounds, this study aims to evaluate the impact of nitrite reduction on geometrical, colour, chemical, microbiological, and sensory traits of dry sausage traditionally produced with red paprika powder.

2. Materials and Methods

2.1. Sausage Production and Sampling. In total, three batches of *kulen* (50 kg each) were made in triplicate using 80% of pork ham and 20% of pork back fat. The control (N110) batch contained 110 mg/kg of sodium nitrite. The second batch (N55) was produced with 50% nitrite reduction (55 mg/kg of $NaNO_2$), while the third (NF) batch was produced without nitrites. Frozen pork back fat was cut in bowl cutter KU 130 AC (Laska, Traun, Austria) and mixed for 3 minutes with previously grounded (6 mm) pork ham, salt (2.2%), red hot paprika powder (1%), glucose (0.5%), and commercial starter culture mixture cT salami fast (CreaTec GmbH, Friedrichshafen, Germany) composed of *Lactobacillus* spp., *Pediococcus pentosaceus*, *Staphylococcus carnosus*, and *Staphylococcus xylosus*. The batter was stuffed into 55 mm diameter collagen casings using vacuum filler VF616 (Handtmann, Biberach an der Riss, Germany). After a resting day, sausages were traditionally cold smoked for 2 days as previously described by Simunovic et al. [9] and transferred to a ripening chamber where they were kept until the end of production under the following conditions: 3 days (23°C, 90%–95% RH), 4 days (20°C, 85% RH), 8 days (18°C, 80% RH), and 23 days (12–15°C, 70% RH). Samples for the analyses were taken on the production day, after 8, 16, 24, 32, and 40 days of manufacturing. At the end of the production, all the samples were vacuum packed and left in a cooling chamber at 4°C. To evaluate the shelf life of the sausages, sampling was performed after 50 and 100 days of storage.

2.2. Computer Vision System (CVS) Analysis. Parameters such as lightness (L^*), redness (a^*), and yellowness (b^*) of the surface, meat, and fat parts of the sausage were all measured independently using CVS as proposed by Tomasevic et al. [22] with some modifications. The colour of the sausage surface was measured before removing the casing to simulate the appearance of the sausage in retail. Afterwards, casings were removed and sausages were cut into 10 mm thick slices, placed on a white plastic tray, and photographed. Images were processed in Colour Checker Camera Calibration v2.2.0 (X-Rite Inc., Grand Rapids, MI, USA) and Adobe Photoshop 2020 (Adobe Inc., San Jose, CA, USA) using 11×11 pixel size colour sample tool.

2.3. Three-Dimensional (3D) Laser Imaging Analysis. Scanning of samples was performed using EinScan-SP (Shining 3D Tech., Hangzhou, China) scanner. Samples were placed on a rotation table and scanned in a dark room as recommended by the manufacturer. Scanning was performed using texture mode while adjusting the brightness depending on the colour of each sample. The duration of one scan was approximately 40 min. The meshing of data was conducted using EXScan S_v3.0.0.1 (Shining 3D Tech., Hangzhou, China) software and a PC with the following configuration: intel core i5-73000HQ CPU, 2.5 GHz, 24 GB RAM, and GeForce GTX 1050 2 GB graphic card. High detail and watertight meshing modes were used to generate 3D models from which the volume (m^3) of the sausage was

calculated. Shrinkage of sausages was expressed as a percentage (%) of the initial volume of the sausage. At each processing stage, the volumes of random sausages from the same batch were obtained using both the water displacement method and 3D laser imaging to calculate relative error (%) of 3D estimated volume as recommended by Zhang et al. [23].

2.4. Physicochemical Analysis. Water activity (a_w), pH, moisture, and biogenic amine contents were determined as previously described by Simunovic et al. [9]. Nitrite and nitrate levels were measured according to EN 12014–4: 2005 ion-exchange chromatography method [24], using 858 Professional Sample Processor autosampler (Metrohm, Herisau, Switzerland) and 930 Compact IC Flex with Oven/SeS/PP system consisting of column oven, sequential suppression, peristaltic pump, built-in degasser, and conductivity detector (Metrohm, Herisau, Switzerland). For ion separation, METROSEP C4 250/4 column (Metrohm, Herisau, Switzerland) was used. Free fatty acids (FFA) content was calculated according to ISO 660:2009 method [25].

2.5. Microbiological Analysis. Upon receipt of samples to the laboratory, sausages were aseptically transferred (triplicates of 10 g) to sterile plastic pouches and 10-fold diluted with Buffered Peptone Water (BPW) (Oxoid, Basingstoke, UK). Homogenisation was performed using Stomacher 400 (Seward Medical, London, UK) for 1 minute. A series of 10-fold dilutions by mixing 9 mL of BPW and 1 mL of the previous dilution was made. Total viable count (TVC) was counted on plate count agar (PCA; Oxoid, Basingstoke, UK) in accordance with ISO 4833-1:2013, while lactic acid bacteria (LAB) was determined according to ISO 15214 : 1998.

2.6. Oral Processing Analysis. Oral processing analysis was conducted in accordance with the study of Djekic et al. [26], with some modifications. A total of eight consumers took part in the test and were served with portions of around 200 g of sausage from each batch and a kitchen knife placed on a plastic cutting board. They were asked to cut three slices of sausage and eat while they were recorded by a video camera. The weight of each portion was measured before and after mastication using technical balance. Obtained videos were afterwards analysed by two researchers and were used to obtain the following oral processing parameters: number of chewing strokes, consumption time of one bite (s), chewing rate (chew/s), eating rate (g/s), and average bite size (g).

2.7. Temporal Dominance of Sensations (TDS). TDS analysis was conducted according to Djekic et al. [27], with some modifications. Selected sensations were hardness, meat flavour, juiciness, soft, fattiness, paprika flavour, and spiciness. Each of the six panelists received three 5 mm thick slices of sausages from each batch. They were instructed to click the start button on a tablet computer at the moment of the first bite and to click the stop button at the moment of swallowing. During analysis, panelists were free to choose the most dominant sensation at the moment and were able to choose the same sensation several times.

2.8. Statistical Analysis. Mean values, standard deviation, testing of normal distribution (Kolmogorov–Smirnov), homogeneity of variance (Levene), and one-way ANOVA with Tukey HSD post hoc test ($P < 0.01$) were conducted using SPSS 23.0 (IBM, Armonk, NY, USA). Correlation between variables was tested using Pearson's linear correlation coefficient ($P < 0.01$). Data obtained by TDS were analysed using MS Excel (Microsoft, Redmond, WA, USA).

3. Results and Discussion

3.1. Colour. The analysis of variance showed no significant ($P < 0.01$) difference between L^*, a^*, and b^* values among analysed batches for *kulen* surface, meat, and fat parts which were all measured independently (Tables 1 and 2). These results are not in line with the results of Szymański et al. [28], who found that reduction of nitrite had a negative effect on colour of cured pork. This can be explained as the colour of *kulen* is greatly affected by the red paprika powder that is naturally rich in red pigments [16]. The research outcome demonstrated that level of paprika powder used in this study was high enough to prevail over the effect of nitrites on the colour of *kulen*. The two most common analytical methods to measure colour of food samples are traditional colorimeter and CVS [29]. Recently, the study by Tomasevic et al. [22] revealed that traditional colorimeters might not be a suitable technique for measuring the colour of bi-coloured meat products like fermented sausages because the aperture size of most colorimeters is too big to measure the colour of meat and fat segments separately. Hence, there is a lack of data in the literature regarding colour parameters of meat and fat parts of fermented sausages that were measured independently, especially in the case of sausages produced with red paprika powder like *chorizo* and *kulen*.

3.2. 3D Laser Imaging. Experimentally determined relative error of volume estimation using 3D imaging was in the range between 2.1% and 4.8%. These values are similar to those found by Zhang et al. [23] but higher than those reported by Goni et al. [30]. In the present study, relative errors of volume estimation were higher at the end of the ripening, which may be due to the formation of an uneven surface area of the sausage that is most pronounced at the end of the process. However, nitrite reduction showed no significant ($P < 0.01$) effect on shrinkage among analysed batches (Tables 1 and 2; Figure 1). Shrinkage showed a very strong positive correlation ($P < 0.01$) with weight loss ($r = 0.99$). On the other hand, shrinkage was negatively related to moisture content ($r = -0.98$) and a_w ($r = -0.93$), which means that moisture evaporation that occurs during ripening may be the main reason for the volume reduction of dry sausages. These results indicate the possibility of weight

Table 1: Changes in colour and chemical traits of *kulen* formulated with different levels of sodium nitrite during the first 16 days of production (mean values and standard deviation).

		Processing time (days)								
		0			8			16		
		N110	N55	NF	N110	N55	NF	N110	N55	NF
Shrinkage (%)					20.28 ± 2.18^{1a}	19.62 ± 2.09^{1a}	20.07 ± 1.72^{1a}	27.92 ± 2.32^{1b}	27.91 ± 2.65^{1b}	27.71 ± 2.45^{1b}
Colour parameters										
Meat	L*	54.43 ± 2.64^{1a}	53.86 ± 3.02^{1a}	54.28 ± 3.73^{1a}	47.57 ± 2.57^{1b}	48.29 ± 3.73^{1ab}	48.43 ± 4.03^{1ab}	45.14 ± 2.34^{1bc}	46.28 ± 5.56^{1bc}	46.86 ± 1.57^{1bc}
	a*	54.71 ± 2.75^{1a}	54.86 ± 2.27^{1a}	52.86 ± 3.29^{1a}	55.14 ± 2.61^{1a}	54.71 ± 2.06^{1a}	54.86 ± 3.80^{1a}	51.86 ± 3.62^{1a}	51.57 ± 4.16^{1a}	50.57 ± 2.30^{1a}
	b*	50.86 ± 6.20^{1a}	49.71 ± 7.20^{1a}	48.86 ± 2.97^{1a}	50.29 ± 4.50^{1a}	49.14 ± 5.11^{1a}	49.57 ± 2.07^{1a}	49.57 ± 3.36^{1a}	49.43 ± 5.80^{1a}	50.86 ± 4.22^{1a}
Fat	L*	74.14 ± 2.08^{1a}	75.86 ± 3.62^{1a}	74.29 ± 2.16^{1a}	77.15 ± 1.68^{1a}	80.14 ± 2.27^{1a}	79.28 ± 2.36^{1bc}	75.14 ± 2.91^{1a}	78.57 ± 1.13^{2a}	77.14 ± 1.06^{12ab}
	a*	20.57 ± 2.22^{12a}	18.43 ± 3.02^{1a}	19.14 ± 3.08^{2a}	18.86 ± 3.53^{1a}	16.14 ± 3.07^{1a}	14.86 ± 2.85^{1b}	18.86 ± 1.57^{1a}	17.14 ± 1.86^{1a}	16.86 ± 1.57^{1b}
	b*	18.86 ± 3.41^{12a}	19.71 ± 3.88^{1a}	18.29 ± 2.13^{2a}	20.86 ± 5.95^{1a}	19.71 ± 3.34^{1a}	17.43 ± 2.46^{1b}	20.29 ± 3.81^{1a}	18.14 ± 2.85^{1a}	21.71 ± 4.35^{1b}
Casing	L*	36.00 ± 3.32^{1a}	37.29 ± 2.21^{1a}	36.29 ± 2.36^{1a}	19.86 ± 2.03^{1b}	20.00 ± 2.77^{1b}	19.86 ± 2.11^{1b}	16.14 ± 3.02^{1b}	16.57 ± 2.37^{1b}	17.71 ± 2.43^{1b}
	a*	46.57 ± 3.73^{1a}	48.29 ± 2.43^{1a}	43.86 ± 2.54^{1a}	36.86 ± 1.57^{1b}	34.57 ± 3.60^{1ab}	35.57 ± 2.30^{1b}	29.14 ± 2.91^{1b}	29.86 ± 3.44^{1bc}	30.57 ± 3.55^{1bc}
	b*	45.43 ± 5.25^{1a}	43.00 ± 3.32^{1a}	42.57 ± 5.56^{1a}	30.57 ± 3.21^{1bc}	27.29 ± 4.39^{1b}	28.43 ± 3.50^{1b}	24.71 ± 4.68^{2b}	23.14 ± 4.84^{2b}	21.86 ± 6.59^{2b}
Chemical parameters										
Nitrites (mg/kg)		90.20 ± 8.48^{1a}	49.10 ± 5.63^{2a}	0.70 ± 0.17^{3a}	38.35 ± 3.26^{1b}	28.50 ± 2.43^{2b}	0.83 ± 0.24^{3a}	21.15 ± 0.17^{1c}	4.50 ± 0.41^{2c}	0.80 ± 0.17^{3a}
Nitrates (mg/kg)		6.43 ± 0.21^{1a}	6.53 ± 0.28^{1a}	6.32 ± 0.16^{1a}	6.39 ± 0.54^{1a}	6.48 ± 0.42^{1a}	6.18 ± 0.02^{1a}	7.43 ± 0.33^{1b}	7.16 ± 0.50^{1ab}	6.22 ± 0.02^{1a}
pH		5.47 ± 0.00^{1a}	5.43 ± 0.01^{1a}	5.48 ± 0.00^{1a}	5.03 ± 0.01^{1b}	5.00 ± 0.02^{1b}	5.04 ± 0.01^{1b}	4.90 ± 0.00^{1c}	4.95 ± 0.01^{2bc}	4.96 ± 0.01^{2c}
a_w		0.945 ± 0.00^{1a}	0.947 ± 0.00^{1a}	0.956 ± 0.01^{1a}	0.929 ± 0.00^{1b}	0.933 ± 0.01^{1b}	0.929 ± 0.01^{1b}	0.923 ± 0.00^{1c}	0.920 ± 0.01^{12c}	0.918 ± 0.01^{2b}
Moisture (%)		58.21 ± 0.58^{1a}	56.01 ± 0.12^{2a}	57.56 ± 0.56^{1a}	52.91 ± 0.28^{1b}	51.65 ± 0.35^{2b}	51.87 ± 0.34^{12b}	46.45 ± 0.17^{1c}	44.83 ± 0.96^{2c}	45.29 ± 0.16^{2c}
Weight loss (%)					19.72 ± 0.35^{1a}	19.68 ± 0.69^{1a}	19.76 ± 0.60^{1a}	28.44 ± 0.42^{1b}	27.99 ± 0.66^{1b}	28.11 ± 0.26^{1b}
Free fatty acids (mg/g lipid)		1.04 ± 0.04^{1a}	1.02 ± 0.02^{1a}	0.71 ± 0.01^{2a}	1.11 ± 0.02^{1a}	0.99 ± 0.02^{2a}	1.24 ± 0.04^{3b}	2.79 ± 0.01^{1b}	2.61 ± 0.02^{2b}	2.87 ± 0.02^{3c}

N110, 110 mg/kg of $NaNO_2$; N55, 55 mg/kg of $NaNO_2$; NF, nitrite-free.
[1–3]Mean values in the same row (corresponding to the same day of ripening) not followed by a common number differ significantly ($P < 0.01$).
[a–f]Mean values in the same row (corresponding to the same batch) not followed by a common letter differ significantly ($P < 0.01$).

Table 2: Changes in colour and chemical traits of *kulen* formulated with different levels of sodium nitrite during the last 16 days of production (mean values and standard deviation).

		Processing time (days)								
		24			32			40		
		N110	N55	NF	N110	N55	NF	N110	N55	NF
Shrinkage (%)		30.06 ± 2.70^{1b}	30.32 ± 2.89^{1c}	30.21 ± 3.01^{1c}	34.59 ± 3.42^{1c}	34.88 ± 3.12^{1d}	34.80 ± 3.28^{1d}	36.34 ± 4.15^{1c}	36.01 ± 3.96^{1d}	35.99 ± 2.78^{1d}
Colour parameters										
Meat	L^*	44.28 ± 3.59^{1bc}	43.14 ± 2.97^{1bc}	42.14 ± 3.32^{1bc}	41.14 ± 2.61^{1c}	41.43 ± 4.20^{1bc}	42.29 ± 4.68^{1bc}	41.29 ± 2.07^{1c}	39.57 ± 1.99^{1c}	41.14 ± 2.85^{1c}
	a^*	51.14 ± 3.39^{1a}	51.71 ± 2.93^{1a}	50.28 ± 2.21^{1a}	51.43 ± 4.68^{1a}	51.57 ± 2.44^{1a}	53.14 ± 2.54^{1a}	52.14 ± 6.49^{1a}	53.57 ± 1.99^{1a}	51.14 ± 2.34^{1a}
	b^*	50.14 ± 5.34^{1a}	51.14 ± 2.19^{1a}	49.57 ± 5.25^{1a}	52.71 ± 2.87^{1a}	53.43 ± 3.21^{1a}	51.86 ± 3.62^{1a}	53.28 ± 3.73^{1a}	51.57 ± 7.57^{1a}	52.86 ± 3.29^{1a}
Fat	L^*	74.57 ± 1.99^{1a}	77.29 ± 1.38^{1a}	76.71 ± 2.21^{1ab}	77.29 ± 3.09^{1a}	76.43 ± 0.97^{1a}	77.86 ± 1.57^{1ab}	74.14 ± 3.44^{1a}	75.86 ± 3.39^{1a}	74.28 ± 2.98^{1ac}
	a^*	18.29 ± 2.14^{1a}	18.86 ± 3.48^{1a}	17.14 ± 2.48^{1b}	18.71 ± 4.07^{1a}	20.86 ± 4.84^{1a}	18.71 ± 3.12^{1ab}	20.57 ± 3.31^{1a}	18.43 ± 2.99^{1a}	19.14 ± 1.77^{1ab}
	b^*	17.71 ± 3.68^{1a}	20.57 ± 3.27^{1a}	18.57 ± 3.41^{1b}	19.14 ± 4.10^{1a}	22.86 ± 3.38^{1a}	23.43 ± 5.68^{1b}	18.86 ± 1.86^{1a}	19.71 ± 4.92^{1a}	18.29 ± 4.68^{1b}
Casing	L^*	16.43 ± 4.18^{1b}	17.43 ± 3.70^{1b}	18.00 ± 4.51^{1b}	16.57 ± 3.55^{1b}	18.56 ± 4.22^{1b}	16.14 ± 4.48^{1b}	15.86 ± 3.14^{1b}	16.43 ± 2.16^{1b}	15.71 ± 3.94^{1b}
	a^*	31.00 ± 5.96^{1b}	29.14 ± 5.24^{1bc}	26.86 ± 4.06^{1cd}	30.43 ± 3.60^{1b}	30.29 ± 5.59^{1bc}	30.00 ± 4.04^{1bc}	25.86 ± 1.86^{1b}	25.00 ± 3.05^{1c}	24.14 ± 2.73^{1ad}
	b^*	21.86 ± 4.41^{1b}	20.57 ± 3.59^{1b}	19.43 ± 4.79^{1b}	20.29 ± 3.95^{1bd}	22.43 ± 3.37^{1b}	21.57 ± 4.25^{1b}	18.71 ± 3.40^{1bd}	19.14 ± 3.72^{1b}	19.29 ± 4.47^{1b}
Chemical parameters										
Nitrites (mg/kg)		0.86 ± 0.13^{1d}	0.82 ± 0.26^{1d}	0.82 ± 0.28^{1a}	0.82 ± 0.36^{1d}	0.85 ± 0.32^{1d}	0.80 ± 0.31^{1a}	0.83 ± 0.28^{1d}	0.81 ± 0.32^{1d}	0.84 ± 0.63^{1a}
Nitrates (mg/kg)		7.97 ± 0.06^{1b}	7.39 ± 0.02^{12ab}	6.60 ± 0.02^{2a}	7.96 ± 0.03^{1b}	7.77 ± 0.04^{12b}	6.56 ± 0.03^{2a}	7.84 ± 0.04^{1b}	6.89 ± 0.02^{1ab}	7.01 ± 0.02^{1a}
pH		4.87 ± 0.00^{1d}	4.91 ± 0.01^{2c}	4.92 ± 0.01^{2d}	4.92 ± 0.00^{1e}	4.94 ± 0.01^{2bc}	4.94 ± 0.00^{2cd}	4.95 ± 0.00^{1f}	5.01 ± 0.01^{2b}	5.03 ± 0.01^{2be}
a_w		0.898 ± 0.00^{1d}	0.897 ± 0.01^{2d}	0.904 ± 0.01^{1bc}	0.883 ± 0.00^{1e}	0.877 ± 0.00^{2e}	0.878 ± 0.01^{2cd}	0.865 ± 0.00^{1f}	0.867 ± 0.00^{1f}	0.867 ± 0.00^{1d}
Moisture (%)		43.26 ± 0.16^{1d}	40.63 ± 0.48^{2d}	43.05 ± 0.04^{1d}	38.01 ± 0.91^{1e}	36.95 ± 0.38^{1e}	38.07 ± 0.18^{1e}	34.58 ± 0.32^{1f}	33.79 ± 0.17^{1f}	34.19 ± 0.19^{1f}
Weight loss (%)		31.58 ± 0.53^{1c}	31.27 ± 0.16^{1c}	31.85 ± 0.23^{1c}	36.15 ± 0.11^{1d}	35.84 ± 0.75^{1d}	35.72 ± 0.14^{1d}	37.53 ± 0.35^{1e}	36.91 ± 0.89^{1d}	37.13 ± 0.16^{1e}
Free fatty acids (mg/g lipid)		2.94 ± 0.01^{1c}	2.66 ± 0.03^{2b}	3.03 ± 0.03^{3d}	3.89 ± 0.01^{1d}	3.00 ± 0.02^{2c}	4.99 ± 0.01^{3e}	3.66 ± 0.02^{1e}	3.56 ± 0.05^{1d}	4.13 ± 0.05^{2f}

N110, 110 mg/kg of $NaNO_2$; N55, 55 mg/kg of $NaNO_2$; NF, nitrite-free.
[1–3]Mean values in the same row (corresponding to the same day of ripening) not followed by a common number differ significantly ($P < 0.01$).
[a–f]Mean values in the same row (corresponding to the same batch) not followed by a common letter differ significantly ($P < 0.01$).

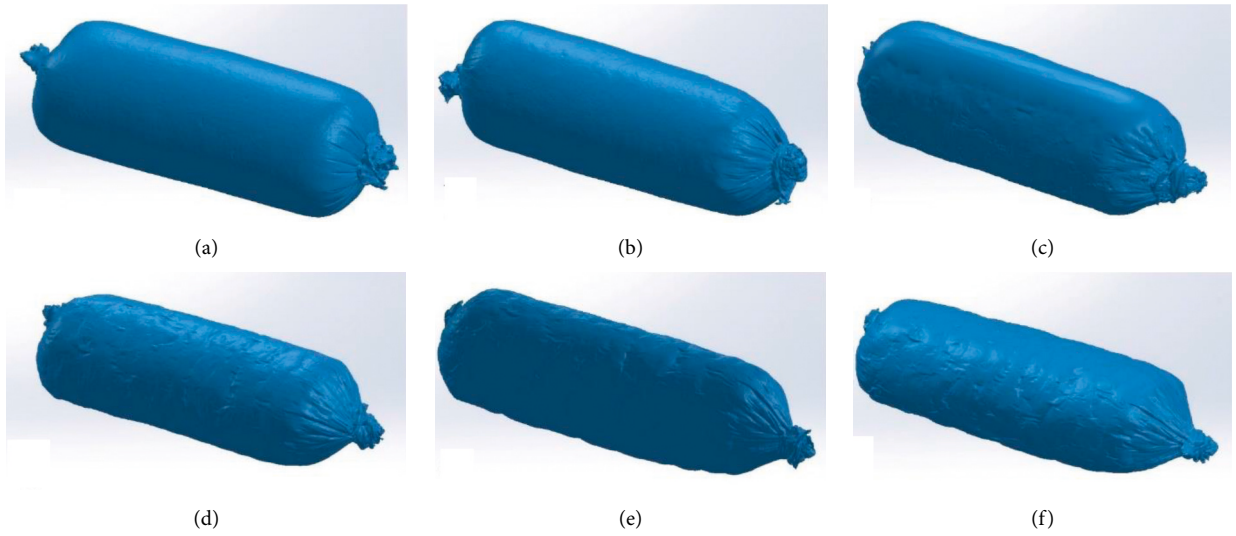

Figure 1: Three-dimensional (3D) models of control (N110) *kulen* during different stages of ripening: (a) production day, (b) 8th day, (c) 16th day, (d) 24th day, (e) 32th day, and (f) 40th day.

loss and moisture content evaluation through shrinkage determination using 3D laser imaging. Moisture content is often defined by meat quality regulations and it represents an important economic parameter in fermented sausage production [9]. Determination of moisture content is time consuming because it involves homogenisation, drying, and cooling of the sample until it reaches constant mass. On the other hand, the application of 3D laser imaging in the estimation of moisture may be a promising alternative to traditional measurements it is relatively fast and non-destructive. This could be particularly important for the meat industry where 3D imaging could be used during different stages of ripening as a tool for the determination of the end of the drying process.

For the industry to apply these measurements, it would be necessary to develop mathematical models for specific types of sausages. In a recent study, Vaskoska et al. [31] investigated the possibility of predicting the cooking loss of pork based on 3D shrinkage measurements and reported relatively inconsistent results. However, the highest correlation ($P < 0.05$; $r = 0.68$) between cooking loss and shrinkage was found to be moderate. According to Uyar and Erdogdu [31], the geometrical characterisation of food to be used in a model is the first step in the development of analytical solutions.

3.3. Chemical Analyses. Nitrite levels significantly ($P < 0.01$) differed among the trials in the first 16 days of ripening during which a gradual decrease in their levels was observed (Tables 1 and 2). Nitrite content in analysed batches was in agreement with the amount of nitrite in sausage formulation. According to Christieans et al. [32], nitrites react quickly dropping their level by more than 50% during the first day of production while at the end of the ripening, their content is usually below 10 mg/kg. This was confirmed by our study in which nitrite levels were found to be around 0.9 mg/kg in the second half of the ripening when no significant differences in their levels were observed among the batches. Contrary to this, levels of nitrates were similar in the first 16 days and kept the trend steadily by the end of ripening in all analysed batches. However, on days 24 and 32, ripening levels of nitrates in N110 batch were significantly higher compared with those found in the NF batch. This could be due to the oxidation of nitrite to nitrate which occurs as a result of remaining oxygen in the sausage [33]. In addition, residual levels of nitrates in *kulen* originate from red hot paprika powder used in this study in which we found 505 mg/kg of nitrates. In European Union, maximum levels of both nitrite and nitrate salts that can be added during the production of fermented sausages are defined by Regulation (EC) No 1333/2008 and are set to 150 mg/kg. An exception to this is Denmark which managed to maintain its more stringent national regulation and set to 100 mg/kg of nitrites [34]. Nitrite reduction had no significant effect on a_w and moisture content. The final a_w of the sausages was around 0.86 and was lower than that reported by Hospital et al. [35] who revealed that reduction and removal of nitrites and nitrates from the formulation of *salchichón* and *fuet* did not compromise safety regarding *C. botulinum* in tested conditions. Moisture content gradually decreased during ripening in all batches showing a strong positive correlation ($P < 0.01$) with a_w ($r = 0.97$). The final moisture content was similar to that found in different sausages by other authors [36, 37].

Free fatty acid content increased during production in all analysed batches (Tables 1 and 2). According to statistical analysis, nitrite-free sausages (NF) contained significantly ($P < 0.01$) higher levels of free fatty acids than that found in batches containing nitrite indicating more intense lipase activity. During ripening, free fatty acids are liberated from triglycerides and phospholipids as a result of hydrolysis by the activity of endogenous and bacterial lipases. Lower levels of free fatty acids in sausages containing nitrites (N110 and N55) could be explained by the antioxidant mechanism of

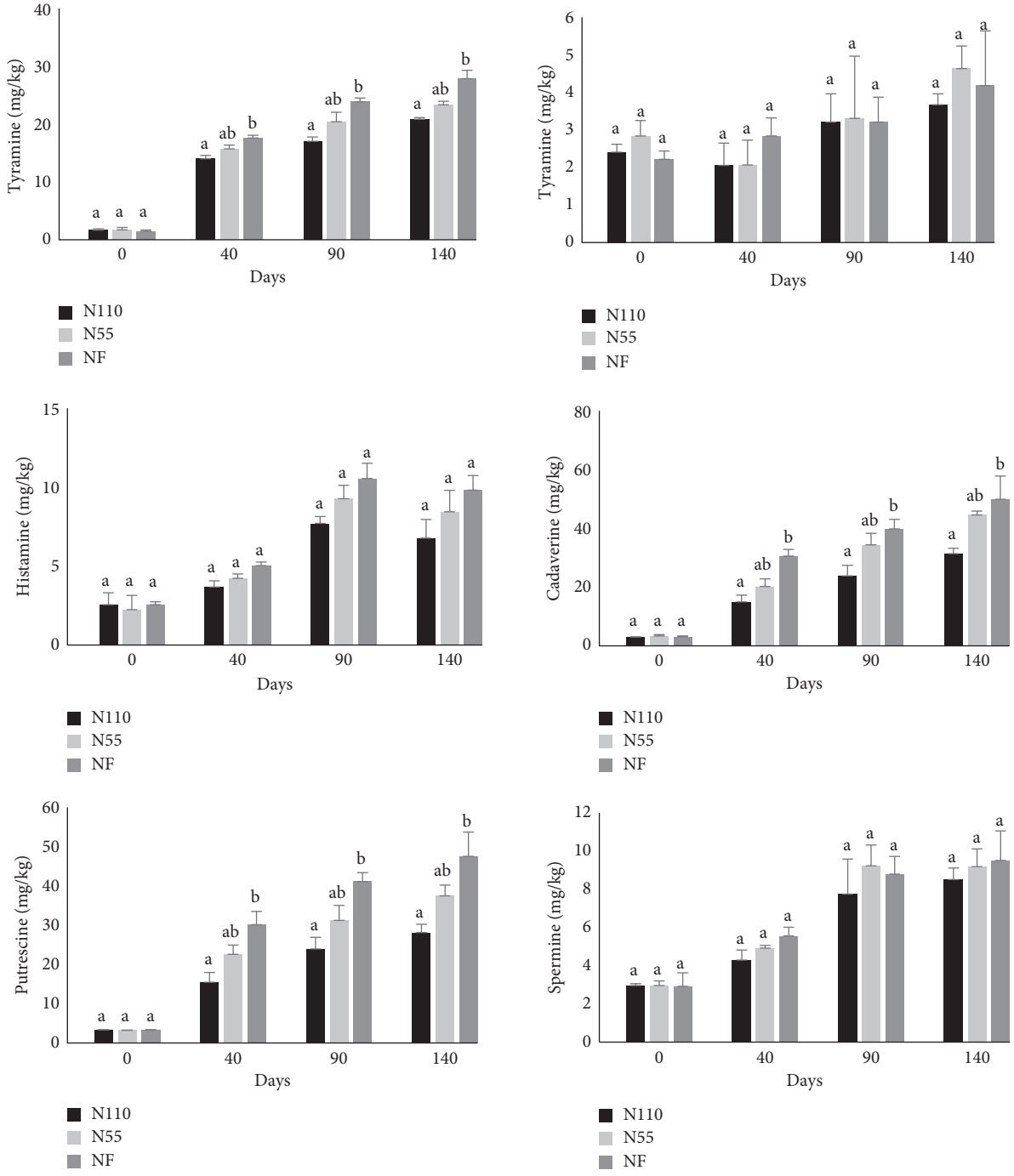

Figure 2: Changes in levels (mg/kg[d]) of tyramine, tryptamine, histamine, cadaverine, putrescine, and spermine in *kulen* affected by sodium nitrite level during ripening and storage (mean ± standard deviation). Values followed by different letters (a–b) differ significantly ($P < 0.01$). [d]Fresh sample weight.

nitrites that involves binding of nitrite oxide to hem iron and other transitional metals whose presence was found to increase lipases production by certain bacterial species for several folds [38–40]. Furthermore, the presence of these metallic ions that are naturally found in meat, may enhance the activity of certain lipases [41]. Importantly, nitrite-free sausages had the highest pH among analysed batches that is closest to the optimum activity of many microbial lipases (pH from 6 to 9) [42, 43].

3.4. Biogenic Amines and Microbial Counts. The increase in pH during ripening is a result of proteolysis and the formation of peptides, ammonia, amino acids, and biogenic amines [2, 9]. Biogenic amine levels are an important food safety parameter as some of them are reported to be hazardous to humans if consumed at critical levels [44]. Contents of tyramine, tryptamine, histamine, cadaverine, putrescine, and spermine gradually increased throughout the ripening and storage in all batches (Figure 2). Nitrite

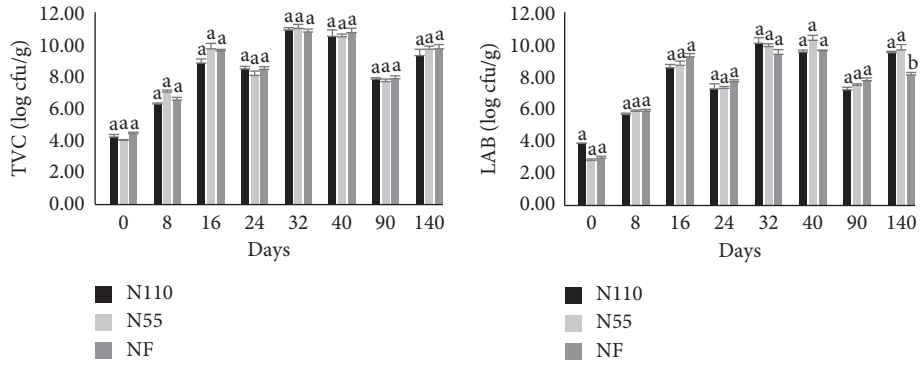

Figure 3: Influence of sodium nitrite reduction on number (log cfu/g) of total viable count (TVC) and lactic acid bacteria (LAB) during ripening and storage of *kulen* (mean ± standard deviation). Values followed by different letters (a–b) differ significantly ($P < 0.01$).

reduction significantly affected ($P < 0.01$) lower levels of putrescine, cadaverine, and tyramine in nitrite-free sausages compared with those formulated with nitrites. Our results are in agreement with those reported by Kurt and Zorba [45], who found that the formation of cadaverine and tyramine significantly ($P < 0.01$) decreased by increasing nitrite content in dry sausages. Although Kurt and Zorba [45] reported lower levels of putrescine in sausages formulated with nitrites, these differences were not found to be significant. The study by Steinberger and Westheimer revealed that the presence of certain cations (Fe^{2+}, Fe^{3+}, and Cu^{2+}) promotes the decarboxylation of some amino acids by decarboxylase activity [46]. Therefore, lower levels of biogenic amines in sausages containing nitrite could be explained by the same mechanism that nitrites express in preventing lipid oxidation [39]. The binding of nitric oxide with these cations may have influenced reduced enzyme activity and consequently resulted in lower levels of biogenic amines in nitrite formulated sausages. However, no significant ($P < 0.01$) differences in levels of biogenic amines were observed between sausages produced using 110 mg/kg of sodium nitrite and those formulated with 55 mg/kg of sodium nitrite.

Biogenic amines are formed by the decarboxylation of amino acids by decarboxylases present in some bacterial species. Therefore, increased biogenic amine production is often related to an elevated number of TVC, LAB, *Enterobacteriaceae*, and enterococci [45]. In the present study, no significant ($P < 0.01$) differences in TVC and LAB counts were found between analysed batches of *kulen* with an exception on 140th day of production (100th day of storage) when significantly lower counts of LAB were observed for nitrite-free sausages than those formulated with nitrite (Figure 3). At the same time, TVC counts among the batches were similar. Considering that LAB is the most dominant flora in TVC, it can be stated that counts of non-LAB bacteria in nitrite-free sausages were the highest. However, this difference was observed only at the end of the storage and does not provide an explanation for elevated biogenic amine levels at the end of the ripening period. The results of our study are in accordance with those reported by Christieans et al. [32] who found no significant differences in LAB counts between the sausages produced with different levels of nitrite. At the end of ripening, counts of TVC and LAB were similar to that reported by other authors for different types of fermented sausages [32, 35–37].

3.5. pH Value. In all samples, the pH value rapidly dropped from the initial value of around 5.45 to 5.00 during the first 8 days of ripening (Tables 1 and 2). This decrease could be due to the accumulation of organic acids, mainly lactic, produced during bacterial fermentation as indicated by many studies [32, 35, 47]. The lowest pH (4.90) was observed on 24th day of the ripening after which it gradually increased to around 5.0 at the end of production. The evolution of pH during ripening was similar to that found for different sausages by other authors [9, 32, 35]. Nitrite levels significantly ($P < 0.01$) affected the pH value of sausages which was found to be the lowest in sausages formulated with 110 mg/kg of sodium nitrite (N110). The results of pH measurements are congruent with levels of biogenic amines as their production is believed to influence the increase of pH sausages in the second stage of ripening. To support this, the pH value of sausages showed a moderate to strong correlation ($P < 0.01$) with levels of tyramine ($r = -0.78$), tryptamine ($r = -0.77$), histamine ($r = -0.87$), cadaverine ($r = -0.89$), putrescine ($r = -0.96$), and spermine ($r = -0.64$). The results of our study are in agreement with those reported by Pennisi et al. who reported significantly lower values of pH in sausages formulated with 150 mg/kg of nitrites than that found in nitrite-free batch [48].

3.6. Oral Processing. The results of the oral processing analysis are shown in Table 3. Removal of nitrite from sausage formulation resulted in a significant ($P < 0.01$) decrease in the number of chewing strokes and consumption time of one bite. According to Peyron et al. [49], the number of chewing strokes and consumption time of confectionery products increase with the hardness of the sample that is in accordance with our previous study [50]. As the number of chewing strokes and consumption time of nitrite-free sausages were lower than that found for sausages formulated with sodium nitrite, it could be assumed that the hardness of NF sausages was the lowest. Consumption time of one bite was strongly related

Table 3: Oral processing parameters of dry sausages formulated with different nitrite levels (mean ± standard deviation).

	N110	N55	NF
Number of chewing strokes	22.79 ± 2.23^{1}	22.37 ± 2.45^{1}	20.62 ± 0.77^{2}
Consumption time of one bite (s)	17.04 ± 2.03^{1}	16.83 ± 2.58^{1}	14.83 ± 1.05^{2}
Chewing rate (chew/s)	1.34 ± 0.08^{1}	1.34 ± 0.11^{1}	1.39 ± 0.07^{1}
Eating rate (g/s)	0.45 ± 0.05^{1}	0.43 ± 0.04^{1}	0.48 ± 0.08^{1}
Average bite size (g)	7.62 ± 0.51^{1}	7.26 ± 0.96^{1}	7.02 ± 1.10^{1}

N110, 110 mg/kg of NaNO2; N55, 55 mg/kg of NaNO2; NF nitrite-free. Values in the same row not followed by a common number differ significantly ($P < 0.01$).

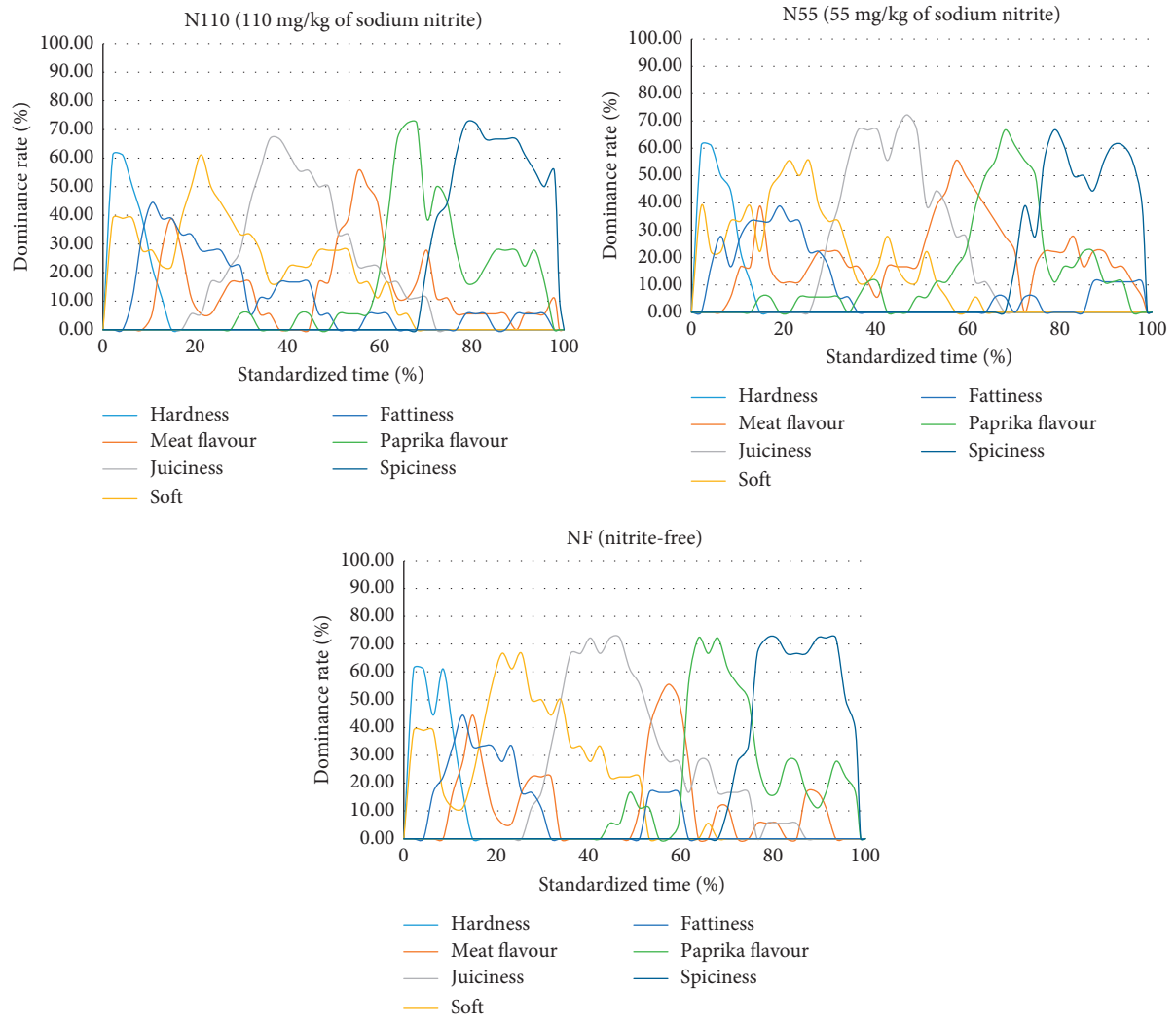

Figure 4: Temporal dominance sensations (TDS) curve of *kulen* formulated with different nitrite levels. $t = 0$ (0% of standardised time; moment of the first bite); $t = 1$ (100% of standardised time; moment of swallowing).

($P < 0.01$) with the number of chewing strokes ($r = 0.88$) and moderately with chewing ($r = 0.72$) and eating rates ($r = 0.51$). According to Aguayo-Mendoza et al. [51], average bite size differs depending on the type of product. In our study, we found no significant differences ($P < 0.01$) between average bite size among analysed batches of *kulen*. In addition, nitrite reduction showed no significant effect on chewing and eating rates. The chewing rate was in accordance with the study of Aguayo-Mendoza et al. [51] who reported a chewing rate of 1.4 chews/s for beef chunk samples. However, our results obtained for eating rate are in disagreement with those reported by Aguayo-Mendoza et al. [51] who found values of around 0.2 g/s. This could be due to the different textures of pork sausage to that found in lean beef which contains higher levels of connective tissue. In addition, in the study of Aguayo-Mendoza et al. [51], there is a lack of data in terms of the culinary method used which is very important as the study of Djekic et al. shows that type of culinary method significantly ($P < 0.05$) affects various oral processing parameters [27].

3.7. TDS. TDS curves for three batches of *kulen* are presented in Figure 4. No significant ($P < 0.01$) differences in TDS curves were observed among the analysed batches. The most dominant sensation in the first 15% of consumption time was hardness, followed by soft sensation and fattiness. Hardness was described as a sensation when high force is required to compress food between the molars, while soft sensation is referred to the small force needed for compression [52]. Fattiness was explained to panelists as a textural attribute that refers to the perception of the quantity of fatty tissue in the body of the product [53]. Our results are in agreement with the study of Lorido et al. [54] who revealed that hardness was a significantly dominant perception in the first 15% of consumption time of dry-cured ham. In our study, juiciness was more dominant (60%–70%) and was perceived earlier (40% of standardised time) compared with the results of Lorido et al. [54]. Juiciness was described as the amount of liquid released from the sample during mastication or the amount of moisture from saliva [55]. This difference could be explained by the different textures of sausages compared with dry-cured ham which may affect earlier excretion and higher saliva incorporation during mastication of samples. The sensation of juiciness was followed by meat flavour which dominated at around 60% of consumption time, while the paprika flavour was perceived as the most dominant between 60% and 75% of standardised time. As a result of red hot paprika in the formulation of *kulen*, the spiciness was dominant in the aftertaste period (last 25% of the time) for 50%–70% of the panel. The spiciness was described to the panelist as a sensation of increased temperature resulting from substances such as capsaicin or hot peppers [53].

4. Conclusion

Nitrite level in *kulen* can be reduced by 50% (55 mg/kg of sodium nitrite) without negatively affecting different quality traits of the product. Sausages produced by reduction of nitrite (N55) showed no significant differences in terms of geometrical, colour, physicochemical, microbiological, and oral processing parameters compared with control (N110) sausages formulated with 110 mg/kg of sodium nitrite. On the other hand, the complete exclusion of nitrites from *kulen* formulation negatively affected the biogenic amines and oral processing properties of the product. Reduction and removal of nitrites from the formulation of *kulen* did not result in significant colour differences in the sausage surface, meat, and fat parts due to high levels of red pigments in paprika powder. TDS curves were identical for all analysed sausages. The limitation of this study is that it does not address the issue of survival of pathogenic microorganisms and the impact of nitrite reduction on oxidative stability. Hence, further studies should be focussed on the effect of nitrite reduction on the survival of pathogenic bacteria, especially *C. botulinum* and shelf life of the product.

Data Availability

The data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, according to the provisions of the contract on research financing in 2021 (No. 451-03-9/2021–14/200050 dated 05.02.2021).

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