

# The Effect of Autochthonous Starter Culture, Sugars and Temperature on the Fermentation of Slavonian Kulen

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## Summary

In this study, the effect of an isolated and well-characterised autochthonous starter culture, glucose and maltodextrin ( $w=0.8\%$ ) and temperatures of 12 and 20 °C on fermentation and quality of Slavonian kulen produced using the traditional technology and recipe were investigated. Physicochemical and microbiological analyses were carried out after 20 days of fermentation. Upon the completion of the production process (90 days), a sensory analysis was carried out. Furthermore, pH value was continuously measured throughout the twenty-day fermentation period. The addition of an autochthonous starter culture and sugars and different fermentation temperatures significantly ( $p<0.05$ ) affected the instrumental colour and texture parameters of the Slavonian kulen. The fermentation was most intense in the samples with added autochthonous starter culture and 0.8 % glucose, and fermented at 20 °C. Microbiological analysis showed that samples with added autochthonous starter culture and fermented at higher temperature contained a higher number of lactic acid bacteria and coagulase-negative staphylococci and were safe. Sensory evaluation confirmed the outcomes of physicochemical and microbiological analyses and showed differences among samples fermented at two different temperatures and with added glucose or maltodextrin and an autochthonous starter culture.

*Key words:* Slavonian kulen, autochthonous starter culture, sugars, fermentation temperature, physicochemical, microbiological and sensory properties

## Introduction

Slavonian kulen is the most representative traditional Croatian fermented pork sausage produced in rural settings using a traditional technology and industrial settings according to a modified recipe (which includes the addition of commercial bacterial starter cultures, nitrate and nitrite salts and isoascorbate). This sausage is traditionally made from the first and second class pork previ-

ously cleaned of connective tissue, damaged parts, blood vessels and fatback, spiked with salt, red hot and sweet paprika powder and garlic, the stuffing ultimately being filled into the pork appendix (*intestinum caecum*). Once the stuffing is conditioned, the sausage is smoked, fermented, dried and ripened for several months (1,2). Due to the various technological processes used for the sausage production, including hurdle technology, the activity of technological

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microflora (especially vivid during the fermentation process) and long-term maturation, complex microbiological, physicochemical and biochemical processes take place resulting in the change of fundamental building materials (fats, proteins and carbohydrates), water loss and increase in dry mass (3).

Nowadays, bacterial starter cultures are widely used in the industrial production of fermented sausages to overcome the fermentation problems, reduce the variability of the product quality, limit the growth of spoilage bacteria by accelerating fermentation, and improve the sensory properties of fermented meat products (4). Fermentation is the crucial phase of dry sausage curing, since in this stage major physical, biochemical and microbiological transformations occur (5,6). Meat conservation by fermentation is characterised by several factors: pH decrease, changes in the initial count of microflora, reduction of nitrates first to nitrites and later on to nitric oxide, formation of nitrosomyoglobin, solubilisation and gelification of myofibrillar and sarcoplasmic proteins, proteolytic, lipolytic and oxidative changes, and dehydration (7). The process is also characterised by the increase in the number of lactic acid bacteria (LAB) from  $10^3$ – $10^5$  to  $10^6$ – $10^9$  CFU/g, as well as by glycolytic sugar degradation and the increase in lactic acid concentration. The increase in lactic acid concentration causes a pH decrease from the initial 5.7 to 5.5 in slow-fermented sausages and to 4.6 (sometimes even 4.2) in fast-fermented sausages (8,9). Bacterial starter cultures used for meat fermentation are the preparations of viable bacteria that exhibit a desired metabolic activity, primarily acidification, and are responsible for the development of aroma during meat fermentation (10). They are composed of the strains of lactic acid bacteria primarily belonging to the *Lactobacillus* or *Pediococcus* genus, as well as of coagulase-negative staphylococci (CNS) and the members of the *Micrococaceae* genus (4).

Fermentation is most intense during the first few hours, when the temperature rises up to the values optimal for LAB growth. This can take from 12 h to 7 days or even longer, depending on the product type, additives, production technology and production environment, *i.e.* temperature and relative air humidity. Higher temperatures and higher relative air humidity (RH) speed up the fermentation process and decrease the pH value. Depending on the bacterial strains, fermentation can be carried out at temperatures ranging from 18–24 °C or higher for 1–2 days, or at lower temperatures (10–12 °C) for over a week. However, in some cases fermentation can last for over a week even if taking place at higher fermentation temperatures (*e.g.* in the production of Greek and some Italian sausages) (11,12).

Since the glucose content in meat is too low or much too variable, and in order to provide sufficient quantities different carbohydrates, like glucose, sucrose, lactose, maltodextrin, corn syrup, starch and sorbitol are added to the fermented sausage stuffing. These carbohydrates enhance the growth of technological microflora, primarily LAB (13). The most common substrate used in this type of fermentation is glucose, consumed during the LAB exponential growth phase.

Fermentation temperature, mass fraction and the type of added sugar directly affect the pH decrease rate.

Low pH inhibits the growth of pathogens and spoilage bacteria, while glucose and salt increase the osmotic pressure, which favours the growth of autochthonous technological bacteria. Sugars, mostly glucose, facilitate dry sausage fermentation, since they serve as a substrate for the lactic acid production and contribute to the specific aroma development. Up to 2 % (on average 0.3–0.8 %) of sugars are added into the fermented sausage stuffing to ensure the pH decrease from the initial 5.8–6.0 to 4.8–5.4 (14).

Inoculation of the sausage stuffing with a starter culture composed of the selected LAB (*i.e.* homofermentative *Lactobacilli* and/or *Pediococci*) and non-pathogenic CNS and/or *Kocuria* improves the quality and safety of the final product and contributes to the standardisation of the production process (13–16).

The goal of this study is to evaluate the impact of an isolated and well-characterised autochthonous starter culture, glucose and maltodextrin ( $w=0.8$  %) and two fermentation temperatures (12 and 20 °C) on the fermentation process and quality of the Slavonian kulen.

## Materials and Methods

### Microorganisms

Starter cultures, bacterial strains *Lactobacillus plantarum* 1K and *Staphylococcus carnosus* 4K1, characterised as functional (1,7–12), are originally isolated from the traditional Croatian fermented sausages. These strains are utilised in an industrial sausage production. Bacterial strains were acquired from the collection of microorganisms stored in the Laboratory for General Microbiology and Food Microbiology, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia. *Lactobacillus plantarum* 1K and *Staphylococcus carnosus* 4K1 were kept at –70 °C in de Man-Rogosa-Sharpe (MRS) broth (Difco™, Detroit, MI, USA) and nutrition broth (Biolife, Milano, Italy) with 30 % (by volume) glycerol. The strains were activated in the above mentioned broths and maintained at 4 °C until propagation.

### Preparation of wet biomass

The LAB were grown in the MRS broth at 30 °C for 48 h, while the *Staphylococcus* was grown in the nutrition broth at 37 °C for 48 h. Bacterial cells were harvested under aseptic conditions as follows: first they were centrifuged (at 6000×g for 10 min) at room temperature, then washed three times in salt water (0.5 %) and finally resuspended in a sterile salt water. A standard dilution method after a 48-hour incubation at 37 °C in MRS and nutrition agar was used to assess the total viable count (TVC). The final counts of bacterial cells were:  $10^{11}$  *L. plantarum* 1K viable bacterial cells per g of wet biomass, and  $10^7$  *S. carnosus* 4K1 viable bacterial cells per g of wet biomass (17).

### Sample preparation

Slavonian kulen was produced in a pilot plant (Faculty of Food Technology, Osijek, Croatia) intended for dry sausage production, equipped with a programmable au-

tomated ripening chamber. The Slavonian kulen stuffing was prepared according to the traditional recipe: pork meat (of the first and second class, 91.8 %), pork fatback (5 %), garlic (0.2 %), red hot paprika powder (0.4 %), sweet red paprika powder (0.6 %) and salt (2 %). The meat was ground through a grinding plate with 8-mm diameter holes, while the pork fatback was ground through a grinding plate with 6-mm holes. The batches with added autochthonous starter culture were inoculated with *L. plantarum* 1K in the final concentration of about  $10^6$  cells per g and *S. carnosus* 4K1 in the final concentration of about  $10^3$  cells per g. To these samples, 0.8 % of glucose or maltodextrin (both from Sigma-Aldrich, Taufkirchen, Germany) were added, as well.

The Slavonian kulen stuffing was stuffed into the pig appendix. After stuffing, the raw samples were cold-smoked using a dry hardwood (hornbeam, beech and its sawdust) every few days (for 3–4 h) for two weeks. A 20-day fermentation process was carried out at two different temperatures (Table 1) and relative humidity (RH) of 80 to 90 %. After smoking and fermentation, all samples were left to rest in the ripening chamber at the temperature from 14 to 17 °C and RH from 70 to 80 %. The entire technological process running under the processing conditions detailed above lasted for 90 days. A total of 15 samples of raw Slavonian kulen were divided into five batches (Table 1).

Table 1. Samples of the Slavonian kulen prepared according to the traditional recipe with the addition of glucose or maltodextrin and fermented with autochthonous starter culture at different temperatures

Batch	<i>t</i> /°C	<i>w</i> (sugar)=0.8 %
B1	20	control
B2	12	glucose
B3	20	glucose
B4	12	maltodextrin
B5	20	maltodextrin

Control=without sugar and autochthonous starter culture; *N*=3

### Physicochemical analysis

The water content was determined gravimetrically (18) at 103 °C (model Epsa 2000; Bari, Velika Gorica, Croatia). The total protein content was determined by the Kjeldahl method (19) using a Unit 8 Basic digestion block (Foss, Hillerød, Denmark) and a Kjeltac 8400 automated distillation and titration device (Foss). The total fat content was determined by the Soxhlet method (20). The sample was submitted to digestion in an acidic environment followed by petroleum ether-induced fat extraction using a Soxtherm 2000 automated device (C. Gerhardt GmbH & CO. KG, Königswinter, Germany). Collagen content was determined by the spectrophotometric analysis of hydroxyproline (21) using a spectrophotometer model DR/4000U (Hach, Düsseldorf, Germany). Titration method was applied in order to determine the salt content (22) using 2 g of each sample. Samples were homogenised

with sand and 3 mL of water and the content was transferred into a 100-mL volumetric flask, stirred and placed into a water bath at 100 °C for 15 min. After cooling, the flask was filled with water up to the mark and filtered. An aliquot (25 mL) of the filtrate was transferred into an Erlenmeyer flask containing a few drops of  $K_2CrO_4$  as an indicator (62 g per 100 mL of water) and titrated with 0.1 M  $AgNO_3$  until a persistent reddish colour was obtained. The volume and concentration of the titration reagent were used to calculate the salt content. A homogenate diluted with distilled water (1:10) was used for pH determination with pH/Ion 510 Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd/ Oakton Instruments, Vernon Hills, IL, USA). The water activity ( $a_w$ ) was determined at room temperature ( $(20 \pm 2)$  °C) using a Rotronic Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland), according to the manufacturer's instructions. Analytical grade chemicals were used for all analyses of physicochemical parameters. For each batch, analyses were done in triplicate.

### Texture profile analysis

Texture profile analysis was performed at room temperature with a Universal TA.XT2i SMS Stable Micro Systems Texture Analyser (Stable Microsystems Ltd., Godalming, UK) equipped with a P/75 cylindrical probe. This involved cutting of samples into 1.5-cm thick slices, subsequently compressed twice to 60 % of their original thickness. Force-time curves were obtained at the crosshead speed of 5 mm/s and the equal recording speed. Determined parameters involved (23): hardness, *i.e.* the maximum force required to compress the sample, springiness, *i.e.* the ability of a sample to recover its original form once the deforming force has ceased, cohesiveness, *i.e.* the extent to which a sample can be deformed prior to rupture, and chewiness, *i.e.* the effort to masticate the sample before swallowing, calculated by multiplying hardness by cohesiveness and springiness. Seven measurements were taken for each sample of the five batches.

### Determination of instrumental colour

HunterLab Mini Scan XE (A60-1010-615 model colourimeter, HunterLab, Reston, VA, USA) was used to determine sample colour (CIE  $L^*a^*b^*$ ). For each measurement, a black standard (light trap) and the standard white ceramic plate ( $L^*=93.01$ ,  $a^*=-1.11$ , and  $b^*=1.30$ ) were applied to standardised the instrument. The Hunter  $L^*$ ,  $a^*$  and  $b^*$  values correspond to lightness (0 (black) to 100 (white)), greenness ( $-a^*$ ) or redness ( $+a^*$ ), and blueness ( $-b^*$ ) or yellowness ( $+b^*$ ), respectively. Colour measurements were performed at room temperature ( $(20 \pm 2)$  °C). For each sample of the five batches, ten measurements were made.

### Microbiological analysis

The determination of microbial population in Slavonian kulen samples employed classical microbiological analyses (Table 2; 24–29). The isolation of microbial populations was carried out according to the microbiological criteria for food (30) and included the isolation of *Enterobacteriaceae*, *Staphylococcus aureus* and sulphite-reducing *Clostridia*, *Salmonella* sp. and *Listeria monocytogenes*.

Table 2. Classical microbiological and biochemical (API) methods used for the isolation and identification of microbial population in Slavonian kulen

Microorganism	Method	Nutrient medium	Incubation conditions		API test
			t/°C	Time/h	
<i>Salmonella</i> sp.	HRN EN ISO 6579:2003/A1:2008 (24)	RP-broth, XLD	37	24–48	API 20 E V4.1
<i>Enterobacteriaceae</i>	HRN ISO 5552:1999 (25)	VRBG	37	24	API 20 E V4.1
<i>Staphylococcus aureus</i>	HRN EN ISO 6888-1:2004 (26)	BP	37	48	API Staph V4.1
Sulphite-reducing clostridia	HRN ISO 15213:2004 (27)	Sulphite agar	37	72	–
<i>Listeria monocytogenes</i>	HRN EN ISO 11290-1:1999/A1:2008 (28)	Fraser broth Palcam agar	37	24	API Listeria V1.2
Lactic acid bacteria	HRN ISO 13721:1999 (29)	MRS agar	30	48–72	API 50 CHL V5.1 API 20 STREP V7.0

After the casing was aseptically removed and discarded, 10 g of a sample were homogenised in 90 mL of sterile 0.5 % saline solution and serially diluted before plating on selective nutrient media (Biolife) (Table 2; 24–29). The incubation took place under aerobic conditions at 37 °C for 48 h. Microbial growth (CFU/g) was determined using the traditional plate counting.

#### Isolation and identification of microbial population

The identity and viability of the applied autochthonous starter cultures and natural microbial population in Slavonian kulen samples were determined using API 50 CH and API Staph identification kits (bioMérieux, Marcy l’Etoile, France). Colonies randomly taken from the MRS (150 colonies) and the Baird Parker agar (100 colonies) (26), descriptive of five samples, were determined using identification kits and then authenticated using a Microflex LT™ matrix-assisted laser desorption-ionisation time-of-flight mass spectrometer (MALDI-TOF MS; Bruker Daltonik, Bremen, Germany) (31). For identification purposes, the peaks from the generated mass spectra were compared to the reference spectra of the integrated database using the MALDI Biotyper software (Bruker Daltonik).

#### Sensory analysis

The final Slavonian kulen samples from all five batches (obtained after 90 days) were subjected to a quantitative descriptive analysis. The analysis was performed by a panel of seven (three male and four female) trained experts according to the ISO standard (32). The panellists had completed three preliminary training sessions in order to familiarise themselves with the involved samples. Twelve attributes were examined and rated on a 5-point scale: 1 standing for poorly perceived or absent and 5 standing for intensely perceived. During these three training sessions, an agreement on the descriptors to be targeted by the analysis was made. This agreement included: two external attributes (appearance and hardness), two attributes describing the slice (colour uniformity and sliceability), five attributes describing the experience during mastication (flavour intensity, juiciness, smokiness, acidic taste and saltiness) and three attributes describing the product smell (spicy odour, lactic acid odour and

mouldy odour). The appearance of Slavonian kulen samples was assessed visually, while the hardness was assessed by palpation. The sliceability as a textural property was assessed visually (to see whether any crumbling is present or whether the pieces of the sausage are falling out). A three-digit code was used for coding of sausage samples. Samples were served cut into approx. 0.4 cm thick slices. In order to cleanse the palate water was offered between the analyses.

#### Statistical analysis

The results are shown as mean value ± standard deviation. Experimental output was processed using the analysis of variance (ANOVA) and the Fisher’s least significant difference (LSD), with the significance defined at the level of probability of 95 % ( $p < 0.05$ ). Statistical analysis was carried out using the Statistica v. 12.7 software (StatSoft Inc. Tulsa, OK, USA).

## Results and Discussion

Physicochemical analysis of the meat and fatback (Table 3) used for Slavonian kulen stuffing showed the mass fraction of the basic building blocks (fat, moisture, proteins and collagen) to be in accordance with the literature data on fresh pork meat and fatback analysed 24 h *post mortem*. The water activity and pH values are also in agreement with the data obtained by the previous research on similar samples (2). A drop in pH values measured 24 h *post mortem* down to below 6 should be observed in meat used for dry sausages, but also for other meat products. This indicates the normal course of *post mortem* glycolysis, lactic acid production and pH value dropdown.

In Slavonian kulen, changes in mass fraction of individual building blocks and water activity ( $a_w$ ) cease after 20 days (Table 4), mostly due to drying. The obtained  $a_w$  of 0.91 was lower than 0.946 and 0.93 reported for a Spanish sausage fermented under similar conditions, but using a different commercial starter culture (5,33). On the other hand, other authors reported the  $a_w$  of 0.88 on day 21 of fermentation and drying of chorizo with added 1 % of glucose and *Lactobacillus sakei* K29 and whose fermentation and drying took place under similar conditions of temperature and relative humidity (34).

Table 3. Basic chemical composition, water activity ( $a_w$ ) and pH of pork fatback, meat and stuffing used for the production of Slavonian kulen

	$w(\text{fat})/\%$	$w(\text{moisture})/\%$	$w(\text{protein})/\%$	$w(\text{collagen})/\%$	$a_w$	pH
Backfat	(74.00±0.06) <sup>a</sup>	(19.93±0.03) <sup>c</sup>	(7.3±0.4) <sup>c</sup>	(2.7±0.1) <sup>a</sup>	(0.96±0.01) <sup>a</sup>	(6.63±0.01) <sup>a</sup>
Meat	(9.00±0.03) <sup>c</sup>	(70.67±0.08) <sup>a</sup>	(19.8±0.4) <sup>a</sup>	(1.4±0.5) <sup>b</sup>	(0.95±0.01) <sup>a</sup>	(5.62±0.01) <sup>b</sup>
Raw stuffing	(12.37±0.04) <sup>b</sup>	(64.3±0.1) <sup>b</sup>	(18.84±0.07) <sup>b</sup>	(1.20±0.03) <sup>b</sup>	(0.94±0.01) <sup>a</sup>	(5.51±0.01) <sup>c</sup>

Values are mean±standard deviation (S.D.),  $N=3$ . Values in the same column marked with different letters in superscript are significantly different ( $p<0.05$ )

Table 4. Basic chemical composition, salt content and water activity ( $a_w$ ) of the Slavonian kulen samples, determined after 20 days of fermentation

Batch	$w(\text{fat})/\%$	$w(\text{moisture})/\%$	$w(\text{protein})/\%$	$w(\text{collagen})/\%$	$a_w$	$w(\text{salt})/\%$
B1	(17.40±0.08) <sup>a</sup>	(54.7±0.4) <sup>b</sup>	(25.1±0.2) <sup>c</sup>	(1.06±0.03) <sup>b</sup>	(0.91±0.01) <sup>b</sup>	(2.73±0.09) <sup>b</sup>
B2	(17.3±0.2) <sup>b</sup>	(53.9±0.2) <sup>c</sup>	(25.2±0.3) <sup>b</sup>	(1.21±0.07) <sup>ab</sup>	(0.91±0.01) <sup>b</sup>	(2.67±0.08) <sup>c</sup>
B3	(17.0±0.1) <sup>c</sup>	(52.61±0.07) <sup>c</sup>	(25.6±0.2) <sup>a</sup>	(1.2±0.1) <sup>a</sup>	(0.91±0.01) <sup>b</sup>	(2.72±0.09) <sup>b</sup>
B4	(16.0±0.2) <sup>c</sup>	(55.18±0.06) <sup>a</sup>	(24.9±0.1) <sup>d</sup>	(1.23±0.05) <sup>a</sup>	(0.91±0.01) <sup>b</sup>	(2.67±0.06) <sup>c</sup>
B5	(16.3±0.4) <sup>d</sup>	(53.11±0.03) <sup>d</sup>	(25.1±0.2) <sup>c</sup>	(1.3±0.3) <sup>a</sup>	(0.92±0.01) <sup>a</sup>	(2.78±0.08) <sup>a</sup>

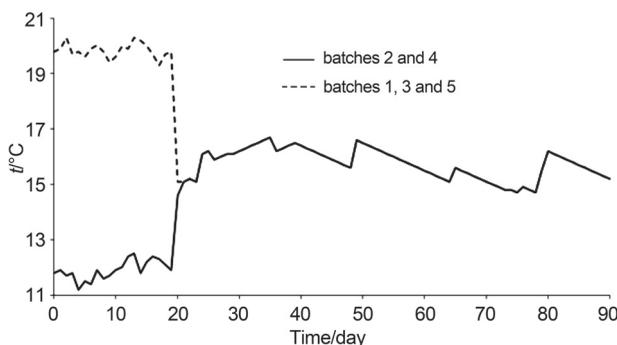
Values are mean±S.D.,  $N=3$ . Values in the same column marked with different letters in superscript are significantly different ( $p<0.05$ )

Temperature and RH in the automated chamber during the production of all five Slavonian kulen batches are presented in Figs. 1 and 2. The RH and temperature were within the limits specified for Slavonian kulen production stages (Table 1).

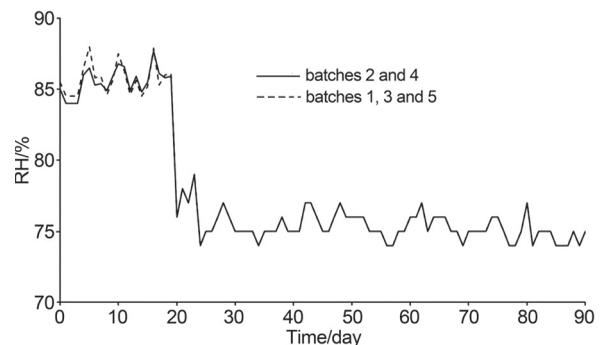
Mass loss determined in all five Slavonian kulen batches is presented in Fig. 3. It is far more substantial in the samples fermented at a higher temperature (20 °C) in the presence of sugars and an autochthonous starter culture, which explains lower moisture contents in these samples (33,34).

During the fermentation phase, the water content in batch B3 decreased from the initial 64.3 % to the lowest 52.6 %. The moisture content was significantly affected by the treatments ( $p<0.05$ ) (Table 4). Samples fermented at higher temperature had lower moisture contents regardless of the addition of sugars and autochthonous starter

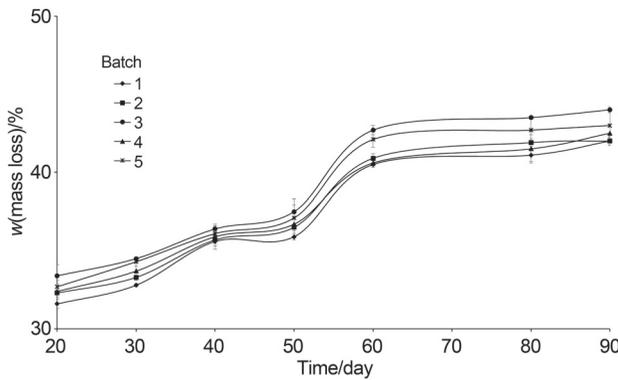
culture. Other studies (33,34) have reported a lower moisture content (47.53 %) in a Spanish dry sausage fermented using a commercial starter culture (*Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus*) at 15 °C and relative humidity of 80 %. In another study (5), a higher moisture content was reported (56.63 %) in the Spanish salchichon fermented using a commercial starter culture (*Pediococcus pentosaceus* and *Micrococcus varian*) and supplemented with 0.45 % sugar (dextrose, lactose and maltodextrin). Moisture content and  $a_w$  variations may be related to the differences in the used microorganisms, the mass fraction and the type of added sugar, and the larger diameter of the Slavonian kulen casing (around 100 mm) than that of the Spanish sausage (60 mm). The salt mass fraction increased from the initial 2 % in raw stuffing to the average value of 2.7 % after 20 days of processing, which complies with the results of similar research in the field (35) (Table 4).



**Fig. 1.** Temperature regime during the production of Slavonian kulen samples: batch 1: without sugar and with autochthonous starter culture, fermented at 20 °C; batch 2: with 0.8 % glucose and autochthonous starter culture, fermented at 12 °C; batch 3: with 0.8 % glucose and autochthonous starter culture, fermented at 20 °C; batch 4: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 12 °C; batch 5: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 20 °C

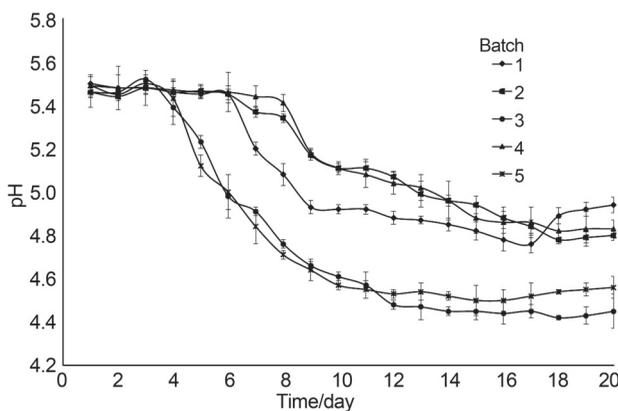


**Fig. 2.** Relative humidity (RH) during the production of Slavonian kulen samples: batch 1: without sugar and with autochthonous starter culture, fermented at 20 °C; batch 2: with 0.8 % glucose and autochthonous starter culture, fermented at 12 °C; batch 3: with 0.8 % glucose and autochthonous starter culture, fermented at 20 °C; batch 4: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 12 °C; batch 5: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 20 °C



**Fig. 3.** Mass losses during the production of Slavonian kulen samples: batch 1: without sugar and with autochthonous starter culture, fermented at 20 °C; batch 2: with 0.8 % glucose and autochthonous starter culture, fermented at 12 °C; batch 3: with 0.8 % glucose and autochthonous starter culture, fermented at 20 °C; batch 4: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 12 °C; batch 5: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 20 °C

Fermentation of dry sausages is usually monitored through changes in pH values (36–38). Changes in the pH values during 20 days of processing in all five batches of the Slavonian kulen are presented in Fig. 4. The results show that in the samples of Slavonian kulen fermented at 20 °C, a substantial decrease in the pH value was seen after 5 days, while in the samples fermented at 12 °C such a decrease was noticed after 7 days. This drop was more intense in the samples fermented at 20 °C and supplemented with an autochthonous starter culture and 0.8 % glucose (batch B3), whose pH dropped from the initial 5.55 to 4.42 after 18 days of fermentation (39) (Fig. 4). The Slavonian kulen fermentation is significantly different from the fermentation of similar Spanish sausages (chorizo). This difference is characterised by a slower pH drop seen in all samples under this study. The lowest pH value of 4.42,



**Fig. 4.** Changes in pH values during 20 days of fermentation of Slavonian kulen samples: batch 1: without sugar and with autochthonous starter culture, fermented at 20 °C; batch 2: with 0.8 % glucose and autochthonous starter culture, fermented at 12 °C; batch 3: with 0.8 % glucose and autochthonous starter culture, fermented at 20 °C; batch 4: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 12 °C; batch 5: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 20 °C

registered in this study in batch B3, was seen on the fermentation day 17, while in the chorizo samples the lowest pH was reached on the ripening day 7 under similar fermentation conditions (34).

As reported by other authors (33), the pH of the Spanish salchichon fermented and dried for 20 days using *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosum* at 15 °C and RH=80 %, equalled to 4.7. Another study (5) reported a similar result (pH=4.87) for a Spanish dry sausage fermented (for 3 days at 25 °C and 90 % RH) using *Pediococcus pentosaceus* and *Micrococcus varians* and subsequently dried for 2 weeks ((15±18) °C, RH=75–80 %).

The decrease in the pH value comes as a consequence of the presence of lactic acid emerging due to the activity of technological microflora (LAB and CNS) and glycolysis, *i.e.* the transformation of sugar into lactic acid (40).

CIE L\*a\*b\* values of the Slavonian kulen samples are shown in Table 5. In fermented sausages, the optimal colour formation strongly depends on the fermentation and the resulting pH decrease and oxygen depletion.

**Table 5.** Colour measurements of the Slavonian kulen samples after 20 days of fermentation

Batch	L*	a*	b*
B1	(47.23±0.07) <sup>c</sup>	(15.5±0.1) <sup>c</sup>	(22.62±0.08) <sup>b</sup>
B2	(50.2±0.2) <sup>b</sup>	(17.1±0.2) <sup>b</sup>	(22.9±0.2) <sup>b</sup>
B3	(50.66±0.03) <sup>a</sup>	(18.6±0.1) <sup>a</sup>	(21.90±0.06) <sup>c</sup>
B4	(49.1±0.1) <sup>c</sup>	(17.28±0.08) <sup>b</sup>	(23.4±0.1) <sup>a</sup>
B5	(48.8±0.1) <sup>d</sup>	(17.47±0.07) <sup>b</sup>	(22.08±0.08) <sup>c</sup>

Values are mean±S.D., N=10. Values in the same column marked with different letters in superscript are significantly different (p<0.05)

The L\* values were significantly modified after the addition of the autochthonous starter culture and sugar, as well as the change of fermentation temperature. The highest L\* values were obtained of the batch B3 samples. Generally, samples fermented at higher temperature had higher L\* values (Table 5). A higher L\* value may be associated with lower pH values and consequently higher lactic acid concentrations established in these samples. In meat, higher lactic acid concentrations cause exudation once the meat proteins have reached their isoelectric point (41). The a\* value was higher in the Slavonian kulen samples fermented at lower temperature. The decrease in a\* value can only be associated with the effect of lactic acid on different myoglobin forms (myoglobin, nitrosomyoglobin and oxymyoglobin). This acid may partially or totally denaturise this compound. Some authors have reported that this acid decreases redness (41). The yellowness was altered markedly (p<0.05) (Table 5) by different fermentation temperatures and the addition of sugars and autochthonous starter culture. Higher fermentation temperature and the addition of autochthonous starter culture decreased yellowness b\* value. This decrease could probably be attributed to the salt content (due to the effect of salt on oxygen solubility in the meat batter)

(41), which was higher in samples fermented at higher temperature in the presence of glucose and maltodextrin and autochthonous starter culture (Table 4).

In view of the above, a more striking downfall of oxy-myoglobin content, which greatly contributes to the value of this colour coordinate, is to be expected. Other authors have also claimed that microorganisms produce metabolites that induce oxidation of meat and fat present in the sausage (42) and, by doing so, contribute to the decrease in this value. Colour parameters measured after 20 days were similar to those of kulenova seka (whose stuffing and production process are virtually the same as for Slavonian kulen, the difference being that the stuffing is stuffed into the end part of the pig large intestine) and the Slavonian homemade sausage (1,2).

The influence of autochthonous starter culture, glucose, maltodextrin and fermentation temperature on the instrumental texture profile (established after 20 days of fermentation) is shown in Table 6. The addition of autochthonous starter culture and sugars and variations in fermentation temperature significantly affected ( $p < 0.05$ ) hardness and chewiness. As expected, hardness and chewiness reached the highest values in batch B3 samples, followed by batch B5 samples, and were the lowest in batch B4 samples (fermented at 12 °C). During fermentation, pH decreased and solubilised myofibrillar proteins aggregated to form a gel.

Therefore, the variability of texture across Slavonian kulen samples seen during fermentation could be explained by pH differences (Fig. 4).

The decrease in the pH gradually induces the aggregation of proteins, leading to the formation of an ordered protein network that contributes to firmness (43).

Table 6. Texture profile of the Slavonian kulen samples after 20 days of fermentation

Batch	Hardness kg	Springiness	Cohesiveness	Chewiness kg
B1	(12.1±0.3) <sup>c</sup>	(0.59±0.08) <sup>b</sup>	(0.59±0.02) <sup>c</sup>	(4.3±0.3) <sup>c</sup>
B2	(11.0±0.8) <sup>d</sup>	(0.54±0.08) <sup>c</sup>	(0.48±0.04) <sup>d</sup>	(2.9±0.9) <sup>d</sup>
B3	(14.2±4.1) <sup>a</sup>	(0.7±0.1) <sup>a</sup>	(0.67±0.04) <sup>a</sup>	(6.6±0.3) <sup>a</sup>
B4	(10.8±0.7) <sup>d</sup>	(0.5±0.1) <sup>c</sup>	(0.48±0.06) <sup>d</sup>	(2.8±0.6) <sup>d</sup>
B5	(13.1±0.4) <sup>b</sup>	(0.6±0.2) <sup>b</sup>	(0.63±0.05) <sup>b</sup>	(5.1±0.3) <sup>b</sup>

Values are mean±S.D.,  $N=7$ . Values in the same column marked with different letters in superscript are significantly different ( $p < 0.05$ )

In addition, acid solubilisation of collagen may occur (44). It has been demonstrated that if the pH falls below the isoelectric point of the muscle protein, solubilisation of the protein is higher producing firmer sausages (45). Our results are in agreement with the above mentioned, since only Slavonian kulen samples supplemented with autochthonous starter culture and 0.8 % glucose and fermented at 20 °C had pH values below 4.5 during fermentation (Fig. 4). A major effect of autochthonous starter culture and sugar addition, and fermentation temperature on springiness and cohesiveness (Table 6) was detected after the end of fermentation.

Generally, batches fermented at higher temperature had higher texture parameter values than samples fermented at lower temperature, irrespective of autochthonous starter culture and sugar addition.

In the Slavonian kulen samples, *Salmonella* sp., *Enterobacteriaceae*, sulphite-reducing *Clostridia* and *L. monocytogenes* were not found (Table 7). Bacterial counts reached at the end of the Slavonian kulen fermentation are shown in Table 5. The highest counts of  $1.2 \cdot 10^7$  of LAB and  $9.6 \cdot 10^3$  CFU/g of CNS were determined in batch B3 samples. In general, higher LAB and CNS counts were established in the samples fermented at higher temperature (Table 7).

The results of biochemical (API) testing of the Slavonian kulen samples (Table 8) showed that the dominant microflora in the samples supplemented with autochthonous starter culture was *Lactobacillus plantarum*, while in the control sample the predominant microflora was *Lactobacillus sakei*. In the samples without autochthonous starter culture, the most represented CNS strains were *S. saprophyticus*, *S. equorum* and *S. warneri*, while in the samples with the autochthonous starter culture the most represented CNS strain was proven to be *S. curvatus* (testing accuracy 99–99.9 %). The dominance of the above strains in the latter samples is to be expected since these strains are the components of the starter culture (17,31,46).

Therefore, we can conclude that the applied autochthonous starter culture has better adapted to the meat environment and to the specific manufacturing process than the indigenous microbial population present in the control sample. In order to avoid possible misclassification, in addition to physiological (API) tests, the identification of the two applied bacterial species (an autochthonous starter culture) was confirmed using MALDI-TOF MS, which corroborated the presence of *L. plantarum* 1K (data not shown) and *S. carnosus* 4K1 (data not shown).

Table 7. Microbiological analysis of the Slavonian kulen samples after 20 days of fermentation

Species	N(CFU/g)				
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Lactic acid bacteria	$6.5 \cdot 10^6$	$5 \cdot 10^5$	$1.2 \cdot 10^7$	$8 \cdot 10^5$	$1 \cdot 10^7$
<i>Enterobacteriaceae</i>	$1.2 \cdot 10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$
<i>Salmonella</i> sp.	N.D.	N.D.	N.D.	N.D.	N.D.
CNS (coagulase-negative staphylococci)	$1 \cdot 10^2$	$1 \cdot 10^2$	$9.6 \cdot 10^3$	$1.2 \cdot 10^3$	$5.1 \cdot 10^3$
Sulphite-reducing clostridia	N.D.	N.D.	N.D.	N.D.	N.D.
<i>Listeria monocytogenes</i>	N.D.	N.D.	N.D.	N.D.	N.D.

N.D.=not determined

Table 8. Biochemical (API) test results for the Slavonian kulen samples obtained after 20 days of fermentation

Batch	Microorganism type	N (colony)	API test	N (microorganism)
B1	LAB	30	<i>L. sakei</i>	21
			<i>L. plantarum</i>	9
			<i>S. saprophyticus</i>	5
B2	CNS	20	<i>S. equorum</i>	6
			<i>S. warneri</i>	9
			<i>L. plantarum</i>	23
B3	LAB	30	<i>L. sakei</i>	4
			<i>S. carnosus</i>	15
			<i>S. warneri</i>	5
B4	CNS	20	<i>L. plantarum</i>	26
			<i>L. sakei</i>	4
			<i>S. carnosus</i>	13
B5	LAB	30	<i>L. sakei</i>	2
			<i>S. carnosus</i>	15
			<i>S. warneri</i>	5

LAB=lactic acid bacteria, CNS=coagulase-negative staphylococci

A complex interaction among physicochemical, biochemical and microbiological processes, playing a role in the formation of chemical compounds, and the modification of molecules responsible for the texture and appearance of the final product, also determine its sensory characteristics. The average scores given by the panellists for all five batches at the end of the Slavonian kulen manufacturing process (90 days) are shown in Fig. 5. As for the external attributes, the highest hardness score was given to batch B3 samples ( $4.4 \pm 0.7$ ), while the appearance of the samples of all five batches was so similar that the assessors could not detect any differences among them. The slicing ability and colour intensity of batch B3 and B5 samples also scored highly.

Regarding the attributes that describe perceptions during mastication, batch B3 samples were highly rated for their flavour intensity ( $4.4 \pm 0.2$ ), juiciness ( $3.8 \pm 0.2$ ) and acidic taste ( $4.3 \pm 0.2$ ). Smokiness and saltiness of all batches were assessed to be similar.

During the fermentation of dry sausages, LAB are known to produce lactic acid (47) responsible for the sour taste (48) and odour of the product, while a mouldy odour is ordinarily associated with 1-octen-3-ol, a typical component of mushroom odour (49). Batches fermented at higher temperature in the presence of glucose or maltodextrin and autochthonous starter culture (Fig. 5) had higher scores for all three attributes.

As for the smell, that of lactic acid was dominant, especially of the batches fermented at higher temperature

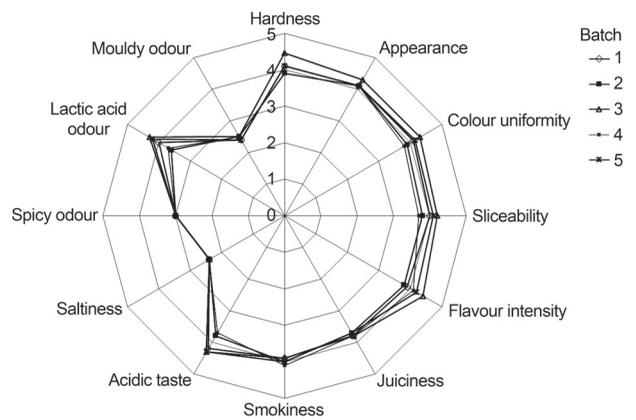


Fig. 5. Mean values of sensory properties of the Slavonian kulen samples determined after 90 days of production: batch 1: without sugar and with autochthonous starter culture, fermented at 20 °C; batch 2: with 0.8 % glucose and autochthonous starter culture, fermented at 12 °C; batch 3: with 0.8 % glucose and autochthonous starter culture, fermented at 20 °C; batch 4: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 12 °C; batch 5: with 0.8 % maltodextrin and autochthonous starter culture, fermented at 20 °C

and supplemented with glucose or maltodextrin and autochthonous starter culture, while mouldy and spicy odour scored low in all five batches.

## Conclusion

The addition of glucose, maltodextrin and autochthonous starter culture and using different fermentation temperatures (12 and 20 °C) affected the Slavonian kulen fermentation. In conclusion, the batches fermented at higher temperature with added autochthonous starter culture had higher values of all textural parameters and  $L^*$  and  $a^*$  values, higher counts of lactic acid bacteria and coagulase-negative staphylococci, and lower moisture,  $a_w$  and  $b^*$  values regardless of sugar addition. Sensory analysis, undertaken at the end of the production process, proved higher fermentation temperature and autochthonous starter culture addition to have the greatest influence on the Slavonian kulen fermentation. Finally, it is important to emphasise that all of the investigated instrumental colour and texture parameters could be useful in monitoring the product evolution and, moreover, in an easy-to-perform control over the total duration of the Slavonian kulen fermentation and ripening.

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