Research article

CAN POLYPHENOLS BE USED AS NATURAL PRESERVATIVES IN FERMENTED SAUSAGES?

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This study was aimed at the investigation into the influence of polyphenols on fermented sausages produced with and without nitrite addition, during storage which lasted for 280 days. Three types of sausages were produced and formed the three experimental groups: C – the control – sausages of usual composition containing nitrites; N+P - sausages with nitrites and polyphenols; and P - nitrite-free sausages with added polyphenols. The proximate chemical composition of all groups was in the range with that of dry fermented sausages. P sausages contained 0.3 mg nitrites per kg, while C and N+P contained 54.8 mg/kg and 52.2 mg/kg, respectively. Polyphenol-enriched sausages had significantly lower peroxide and TBARS values than C sausages. In all sausages lactic acid bacteria counts reached 8.9-9.9 log cfu/g, but decreased during storage to 4.3-4.8 log cfu/g at the end of the storage period. *Micrococcaceae* counts remained stable: 3.5-3.9 log cfu/g. In P and N+P sausages a significantly lower number of *Pseudomonadaceae* was observed than in the control. The lightness of C and P sausages was similar (L=50.2 and L=49.5, respectively), while N+P sausages were darker (L=42.5). C and N+P sausages had similar redness (a*=14.5 and a*=13.2, respectively) and yellowness (b*=5.9 and b*=6.4, respectively), but the values which correspond to redness and yellowness were lower in P sausages (a*=8.0 and b*=4.6). Sensory characteristics of all products were found to be very similar. The flavour of polyphenol-enriched sausages was considered to be better. The most dominant polyphenol in sausages was kaempferol-3-O-glucoside followed by quercetin, luteolin-7-O-glucoside, catechin and syringic acid. Nitrite-free polyphenol-enriched sausages reached the same shelf life as conventional sausages containing nitrites did, which is a promising result implying that polyphenols might be used as natural preservatives and nitrite substitutes. Simultaneous use of nitrite and polyphenols is questionable due to their interactions which should be further studied.

Key words: fermented sausage, nitrite, polyphenols, shelf life

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INTRODUCTION

Fermented sausages are produced from ground meat and fatty tissue, with the addition of table salt, additives, sugar, spices and some other ingredients such as starter cultures, fibers, carbohydrates etc. The technological process of production includes preparation of the stuffing, filling into casings, smoking and drying, followed by ripening that includes physical, chemical and enzymatic processes that enable shelf life and provide sensory properties to the product [1]. Fermented sausages are meat products of high quality and are truly appreciated among consumers. According to the regulations [2], dry fermented sausages should contain less than 30% moisture and more than 20% meat proteins, while the collagen content in meat proteins should be less than 15 %. The safety and shelf life of dry fermented sausages are based on the decrease in pH- and a_w-values during fermentation and drying processes, respectively, which prevents spoilage and growth of pathogenic microorganisms [3-5]. Characteristic sensory properties of the product, such as colour, flavour and texture, are formed through the ripening process. A typical red colour is formed by myoglobin reduction, as well as the formation of nitroso-myoglobin in sausages treated with curing salts containing nitrite and/or nitrate [1]. The flavour is influenced by fermentation, proteolysis and lipolysis, resulting from the activities of sausage microbiota and tissue enzymes, when organic acids, peptides, amino acids, amines, fatty acids, peroxides and aldehydes are released [6-8]. These compounds jointly contribute to the typical flavour of fermented sausages, but free fatty acids are especially prone to oxidation which could lead to the rancidity and spoilage of the product [1,6].

In order to provide microbiological safety, lipid stabilization and the slowdown of oxidation processes throughout the sausage ripening, as well as colour and flavour formation, nitrites play an irreplaceable role in contemporary meat processing. However, nitrites are precursors for harmful N-nitrosamines, which are formed in the reactions between nitrites and amines that are released during sausage ripening [9]. N-nitrosamines are reported as harmful compounds for human's health with a carcinogenic potential [10,11] which prompted research in the direction of reducing the use of nitrites in meat products or finding suitable replacements. However, this is not an easy task due to the multiple significance of nitrite in meat products so an appropriate substitute should act as an antimicrobial, antioxidant, colouring and flavouring agent simultaneously [12].

Polyphenols are secondary metabolites of plants which play important physiological roles protecting them from microorganisms and ultraviolet radiation. These compounds include flavonoids (anthocyanins, flavanols, isoflavonoids, flavonoids, and flavanones) and phenolic acids. It has been proved that they can perform a series of biological effects including antioxidant, antimicrobial, anti-carcinogenic and anti-inflammatory actions [13]. Owing to these properties, the use of polyphenols in meat products could provide a double effect. On the one hand, they could play a role as

natural preservatives, and on the other hand they could act as functional ingredients having a consumer's health promoting potential [14].

The aim of this study was to investigate the influence of polyphenols on physicochemical, chemical and microbiological processes, as well as on sensory properties of fermented sausages produced with and without nitrite addition.

MATERIAL AND METHODS

Sausage production and sampling

The composition of the three experimental groups of fermented sausages was as follows:

– Control group – sausages of usual composition containing nitrites(C): beef meat (35%), pork meat (35%), pork back fat (27%), nitrite curing salt (2.2%), sugar (0,2%), spices (0,2%) and a starter culture.

– The group of sausages containing nitrites and polyphenols (N+P): beef meat (35%), pork meat (35%), pork back fat (27%), nitrite curing salt (2.2%), a polyphenol preparation – powdered grape seeds and skin (0.55%), sugar (0.2%), spices (0.2%) and a starter culture.

– Nitrite-free sausages containing polyphenols (P): beef meat (35%), pork meat (35%), pork back fat (27%), table salt (2.2%), a polyphenol preparation – powdered grape seeds and skin (0.55%), sugar (0.2%), spices (0.2%) and a starter culture.

Sausage stuffing was prepared in a bowl chopper, stuffed into collagen casings 55 mm in diameter, and subjected to smoking, drying and ripening processes, in the following conditions: fermentation - 2 days at a temperature of 26° C and a relative air humidity (RH) 90%; smoking - occasionally for 3 days at 22 to 24° C, drying and ripening at 15° C while RH gradually decreased from 90% to 75% in the following 30 days. The total production process lasted 35 days. The products were stored at a temperature of $+15^{\circ}$ C and RH of 75% during 280 days.

Six sausages were randomly taken from each experimental group and samples were investigated in duplicate. The investigation was conducted during production period (stuffing and the end-product) and during storage on day 0 (at the beginning of storage), 30, 70, 100, 130, 190, 220, 250 and 280.

Physicochemical and chemical analysis

Physicochemical analysis included the determination of water activity (a_w) with an aw-meter (FAst/1, GBX Scientific Instruments) according to ISO, 2004, and pH value measurement with Testo 205 pH meter (Testo AG, Lenzkirch, Germany) according to the reference method [15].

The chemical composition of the sausages was determined by measuring the moisture, protein, hydroxyproline, fat, table salt, ash, nitrite and nitrate contents using standard methods [16-23]. The collagen/protein ratio (the relative content of collagen in meat protein) was calculated as follows: collagen content (%) x 100 / protein content (%).

Lipid oxidation was determined through the acid number [24], peroxide value [25] and TBARS value according to Tarladgis [26] and Holland [27]. Proteolysis index (PI) was calculated according to the method described by Careri et al. [28].

Microbiological analysis

Microbiological investigation included the enumeration of lactic acid bacteria on MRS agar (Merck, Germany) incubated under anaerobic conditions at 30°C for 72 h; *Micrococcaceae* on Mannitol Salt Agar (HiMedia, Mumbai, India) aerobically at 30°C for 48 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (Merck, Germany) at 37°C for 24 h, *Pseudomonadaceae* on CFC Pseudomonas Agar (Oxoid, UK) at 25 °C for 48 h. The results were expressed as logarithms of colony-forming units per gram (log cfu/g). The presence of *Salmonella* spp. and *Listeria monocytogenes* was investigated by standard methods [29,30].

Instrumental colour analysis

The colour of the sausages was determined instrumentally (ChromaMeter CR-400, Minolta Co. Ltd, Tokyo, Japan), using a D-65 light source, a 2° standard observer angle and an 8 mm aperture in the measuring head, according to the CIE L*a*b* system (L* – lightness, a* – redness, b* – yellowness). The results were obtained as the average value of three measurements on the cross-section surface of one sample.

Sensory analysis

Sensory evaluation was made by six panellists trained according to standard procedure [31], by means of the quantitative descriptive analysis according to a 5-point scale with scores from 5 (excellent) to 1 (unacceptable). The sausages with scores 2.0 and higher for each attribute tested (colour, texture, cross section appearance and flavour) were considered acceptable.

Polyphenols content analysis

Polyphenol content determination in the sausage extracts included two steps: the extraction of phenolic compounds and the HPLC–MS–MS analysis.

Extraction of phenolic compounds was performed as follows: 30 g of sausage samples were mixed with 120 mL of methanol and water (80/20, v/v) containing 20 mg/L of butylatedhydroxytoluene (BHT). The system was homogenized using a rod dispenser (T18 Digital Ultra-Turrax, IKA®-Werke GmbH & Co, Germany) for 1 min.

at 6,000 rpm, centrifuged at 4,000 rpm for 10 min and the supernatant was recovered. The operation was repeated twice, and the collected extract was then concentrated by rotary evaporator (Heildolph, Germany) until reaching 50 mL, which was used for the extraction of phenols by solid-phase extraction (SPE). An ODS-C18 SPE cartridge (AccuBOND II ODS-C18, Agilent Technologies, 500mg) previously activated with 10mL of methanol and 10 mL of water was loaded with the obtained water extract. The elution of phenolic compounds was performed with 10 mL of methanol. After solvent removal under vacuum, the phenolic compounds were solubilized in 1 mL methanol and passed through a 0.2-μm-pore-size RC filter (Merck KGaA, Germany). The extract was submitted to HPLC-MS/MS analysis.

HPLC–MS–MS analysis was performed as follows: 15 working standards, ranging from 1.53 ng/mL to $25{,}0 \cdot 10^3$ ng/mL, were prepared by serial 1:1 dilutions of standard mixture with mixture of distilled water and methanol (1:1). Prepared extracts and standards were analysed using Agilent Technologies 1200 Series high-performance liquid chromatograph coupled with Agilent Technologies 6410A Triple Quad tandem mass spectrometer with electrospray ion source, and controlled by Agilent Technologies Mass Hunter Workstation software – Data Acquisition (ver. B.03.01). Five microlitres were injected into the system, and compounds were separated on Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 μm) rapid resolution column held at 50°C. Mobile phase consisting of 0.05% aqueous formic acid (A) and methanol (B) was delivered at flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min). The eluted components were detected by MS, using the ion source parameters as follows: nebulization gas (N2) pressure 50 psi, drying gas (N2) flow 10 L/min and temperature 350°C, capillary voltage 4 kV, negative polarity. Data were acquired in dynamic SRM mode, using the optimised compound-specific parameters (retention time, precursor ion, product ion, fragmentor voltage and collision voltage). For all the compounds, peak areas were determined using Agilent Mass Hunter Workstation Software – Qualitative Analysis (ver. B.03.01). Calibration curves were plotted and sample concentrations calculated using the Microsoft Excel software.

Statistical analysis

Statistical analysis of the results was conducted using the software GraphPad Prism version 6.00 for Windows (GraphPad Software, USA). The two-way analysis of variance (ANOVA) was used to determine the significance of the differences between experimental groups. After significant interactions were found, the data were evaluated by one-factor analysis of variance (ANOVA) with Tukey's multiple comparison test. Statistical significance was considered at the level of $P < 0.05$.

RESULTS

Chemical composition

The results of the investigation into the chemical composition of the sausages are shown in Table 1. At the end of the production, moisture (29.4 – 33.0 %), fat (40.4-42.3 %) and proteins (21.6 – 23.2 %) content, as well as the collagen/proteins ratio (5.9 – 7.7) were very similar among experimental groups. Significant differences were found in the contents of nitrites and nitrates. P sausages contained nitrites in traces (0.3 mg/kg) in the stuffing, which is significantly lower than in C and N+P sausages (54.8 and 52.2 mg/kg respectively). Nitrates were present in the stuffing of P sausages in the amount of 8.7 mg/kg, and in C and N+P sausages the nitrate content ranged between 38.2 and 34.4 mg/kg respectively. In the end-products, both nitrite and nitrate contents lowered compared to the stuffing. P sausages contained 0.2 and 0.3 mg/kg nitrites and nitrates respectively. There was no significant difference in the nitrite content between C (11.4 mg/kg) and N+P sausages, but concerning the nitrates content, N+P sausages had almost twice as high content of nitrates (22.02 mg/kg) as did C sausages (13.78 mg/kg).

Table 1. Chemical composition of the stuffing and end-products after 35 days during the production (Mean ± SD)

Parameter	Stuffing			End-products		
	C	**N+P**	**P**	**C**	**N+P**	**P**
Moisture (%)	54.3 ± 0.7	54.5 ±0.4	53.6 ± 0.3	33.0 ± 0.3	30.3 ± 0.3	29.4 ± 0.2
Fat (%)	26.1 ± 0.2	26.3 ± 0.3	26.7 ± 0.3	40.4 ± 0.3	42.1 ± 0.2	42.3 ± 0.3
Proteins (%)	15.7 ± 0.3	15.6 ± 0.2	15.6 ± 0.2	22.2 ± 0.2	23.2 ± 0.2	21.6 ± 0.3
Collagen/ proteins ratio (%)	7.3 ± 0.4	7.9 ± 0.5	8.5 ± 0.2	5.9 ± 0.1	6.4 ± 0.1	7.7 ± 0.1
Salt (%)	2.6 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	3.5 ± 0.02	3.6 ± 0.03	3.6 ± 0.03
Ash (%)	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1
Nitrites (mg/kg)	54.8 ± 1.5 [a]	52.2 ± 1.3 [a]	0.3 ± 0.03 [b]	11.4 ± 2.4 [a]	12.1 ± 4.7 [a]	0.2 ± 0.1 [b]
Nitrates (mg/kg)	38.2 ± 4.7 [a]	34.4 ± 1.5 [a]	8.7 ± 0.5 [b]	13.78 ± 1.6 [a]	22.02 ± 3.3 [b]	0.3 ± 0.2 [c]

a,b,c = different letter indicates differences (P < 0.05) between experimental groups for investigated parameters separately for the stuffing and the end-products
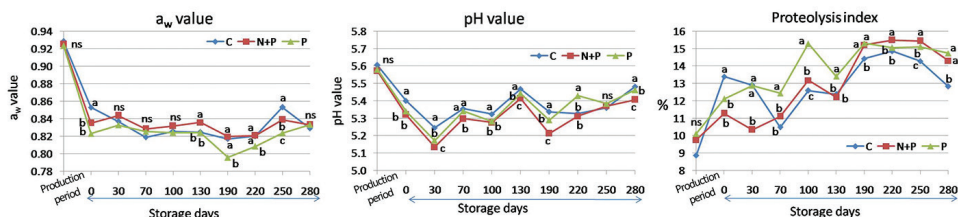C = control - a sausage of usual composition containing nitrites
N+P = sausage produced with nitrites and polyphenols
P = nitrite-free sausage with polyphenols

Physicochemical and oxidative changes

The changes in a_w- and pH- values as well as proteolysis indexes in the fermented sausages are shown in Figure 1.

Figure 1. Changes in a_w, pH and PI in fermented sausages during production and storage
Legend: a,b,c=different letters indicate differences (P < 0.05) between groups on a specific day
ns= difference not significant; C = control - sausages of usual composition containing nitrites
N+P = sausages produced with nitrites and polyphenols; P = nitrite-free sausages with polyphenols

During the 35-day production period, the a_w value declined from 0.92 - 0.93 in the stuffing to the values that ranged from 0.82 (P sausages), 0.83 (N+P sausages) up to 0.85 (C sausages) and remained mainly unchanged during storage. The more intensive a_w value drop was observed in polyphenol-containing sausages (N+P and P), where the lowest values were observed by P sausages after 190 days of storage (0.79) but after 280 days there was no difference between experimental groups (0.83).

The pH value decreased from 5.57 (N+P and P sausages) and 5.61 (C sausages) in the stuffing to 5.32 - 5.34 (N+P and P, respectively) and 5.40 (C sausages) after 35 days of production. During the storage, pH value gradually increased in all sausages, but it was significantly lower in polyphenol containing sausages compared to the control, reaching 5.41 (N+P), 5.46 (P) and 5.48 (C) after 280 days.

Proteolysis index increased from 8.87-10.11 in the stuffing to 12.83-14.73 after 280 days of storage. After the 35 days production period, the highest PI value was observed in C sausages (13.4) compared to P (12.1) and N+P (12.1) sausages. But during the storage period, higher PI values were detected by polyphenol-enriched sausages, where maximal PI values were observed after 100 days (15.28 in P sausages) and after 220 days (15.49 in N+P sausages) of storage.

The results of the investigation into lipid oxidation changes during production and storage of the sausages are shown in Figure 2.
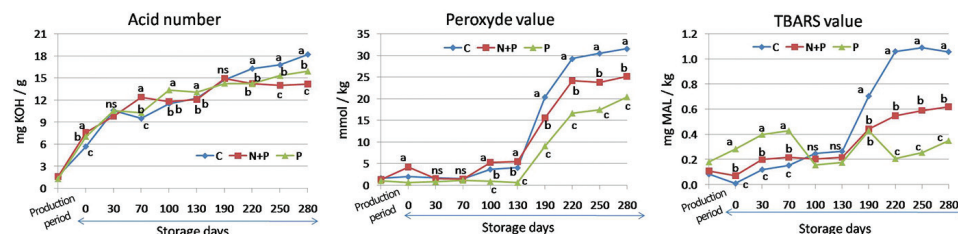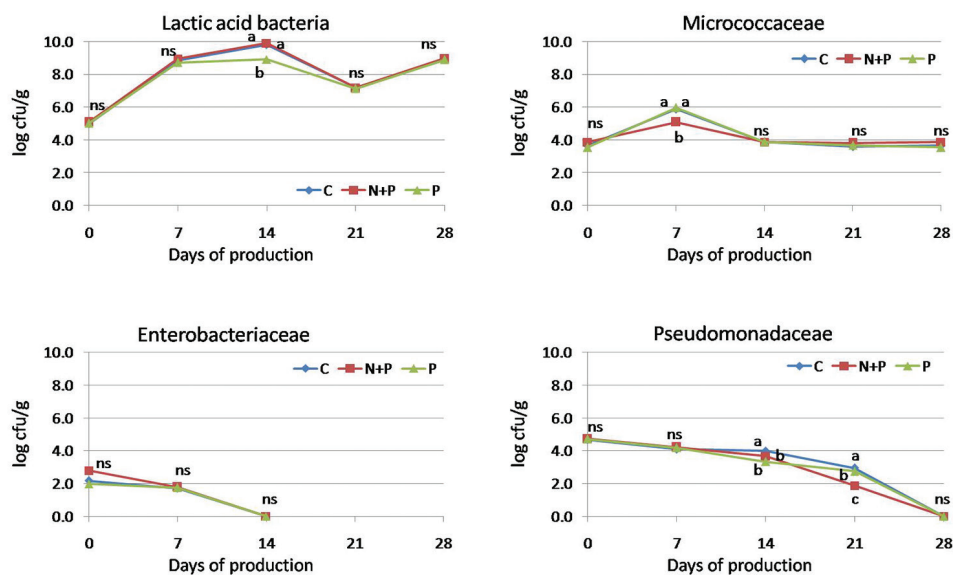

Figure 2. Lipid oxidation parameters of fermented sausages during production and storage
Legend: a,b,c = different letters indicate differences (P < 0.05) between groups on a specific day; ns = difference not significant; C = control - sausages of usual composition containing nitrites; N+P = sausages produced with nitrites and polyphenols; P = nitrite-free sausages with polyphenols

The acid value was higher after the production period in polyphenol-enriched sausages (7.1 mg KOH/g in P and 7.6 mg KOH/g in N+P sausages) compared to the control (5.7 mg KOH/g), such trend remained up to day 190 of storage. Afterwards, the highest acid value was observed in the control group reaching 18.2 mg KOH/g after 280 storage days, while in polyphenols-enriched sausages it ranged between 14.2 (N+P) and 15.9 (P) mg KOH/g. On the other hand, peroxide and TBARS values showed different pattern in the first (up to day 130) and second period of storage (from day 130 to day 280). In the first period, the lowest peroxide value (0.68-1.2 mmol/kg) and highest TBARS value (0.18-0.43 mg MAL/kg) was observed in P sausages. But after 130 days of storage polyphenol-enriched sausages had significantly lower peroxide (9.1-20.4 mmol/kg in P and 15.5-25.1 mmol/kg in N+P sausages) and TBARS (0.21-0.43 mg MAL/kg in P and 0.44-0.60 mg MAL/kg in N+P sausages) values than C sausages (20.4-31,5 mmol/kg and 0.70-1.10 mg MAL/kg respectively). In this period P sausages also had significantly lower peroxide and TBARS values then N+P sausages.

Microbial changes

The results of microbiological investigation during the production of the sausages are shown in Figure 3, and during the storage time in Figure 4.

Figure 3. Microbiota composition in fermented sausages during production
Legend: a,b,c = different letters indicate differences (P < 0.05) between groups on a specific day; ns = difference not significant; C = control - sausages of usual composition containing nitrites; N+P = sausages produced with nitrites and polyphenols; P = nitrite-free sausages with polyphenols
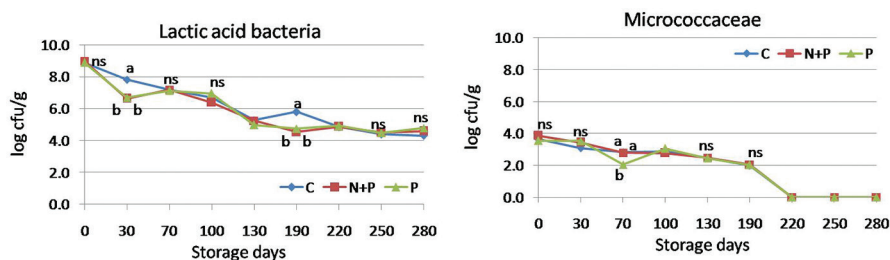
Figure 4. Microbiota composition in fermented sausages during storage
Legend: a,b,c = different letters indicate differences (P < 0.05) between groups on a specific day; ns= difference not significant; C = control - sausages of usual composition containing nitrites; N+P = sausages produced with nitrites and polyphenols; P = nitrite-free sausages with polyphenols

The results showed that the most abundant were lactic acid bacteria (LAB), rising from 5.0-5.1 log cfu/g (in the stuffing) up to 8.9 log cfu/g in P sausages and to 9.8 and 9.9 log cfu/g in C and N+P sausages, respectively, on day 14 of production (Figure 3). There was no difference in LAB counts between experimental groups through the remaining of the storage period. The counts of *Micrococcaceae* was rather similar in all sausages during production (3.5-3.9 log cfu/g), except on day 7, when N+P sausages contained significantly lower numbers (5.1 log cfu/g) than C and P sausages (5.9 and 6.0 log cfu/g, respectively). *Enterobacteriaceae* and *Pseudomonadaceae* which were present in the stuffing in counts of 2.0-2.8 log cfu/g and 2.9-5.2 log cfu/g respectively, were not detected after 14 days and after 28 days of production. In P and N+P sausages a significantly lower number of *Pseudomonadaceae* was observed on days 14 (3.33-3.68 log cfu/g, respectively) and 21 (1,87-2,75 log cfu/g, respectively) compared to the control (3.98 on 14th and 2.94 log cfu/g on day21).

During storage LAB count decreased from 8.9-9.0 log cfu/g to 4.3-4.8 log cfu/g after day 280, being similar in all sausages (Figure 4). Exceptions were observed on days 30 (6.6 log cfu/g in N+P and 6,7 log cfu/g in P sausages) and 190 (4.5 log cfu/g in N+P and 4.7 log cfu/g in P sausages), when LAB counts were significantly lower in polyphenol-containing sausages compared to the control sausages (7.8 and 5.8 log cfu/g, respectively). *Micrococcaceae* count decreased from 3.5-3.9 log cfu/g to 2.0 log cfu/g after 130 days and afterwards they were not detected anymore till the end of the storage time. *Salmonella* spp. and *Listeria monocytogenes* were not detected in any of the production and storage phases.

Colour parameters and sensory evaluation

The results of instrumental colour measurement according to the CIE L*a*b* system are presented in Figure 5. At the beginning of the storage period, C and P sausages had similar lightness (L*= 50.2 and 49.5 respectively), while N+P sausages were significantly darker (L*= 42.5). At the same time, C and N+P sausages had similar redness (a*= 14.5 and 13.2, respectively) and yellowness (b*=5.9 and 6.4, respectively)

while these parameters were significantly lower in P sausages (a*=8.0 and b*= 4.6). At the end of the storage, lightness was similar in all experimental groups on days 250 (L*=46.4-49.9) and 280 (L*=44.7-46.9). Redness was significantly lower in polyphenol-containing sausages (a*=8.0-12.3 in P sausages and a*=10.1-12.0 in N+P sausages) compared to the control (a*=14.5-17.7) during the whole storage period. On the contrary, control sausages had higher yellowness (b*=5.9-7.8) compared to the polyphenol-enriched sausages (b*=4.6-7.1 in P sausages and b*=4.3-6.3 in N+P sausages) during the whole storage period.
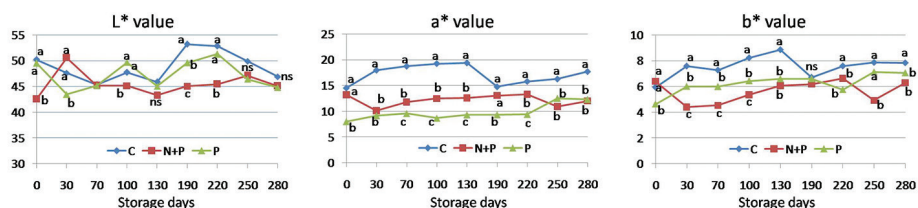
Figure 5. Parameters of instrumental colour analysis of fermented sausages
Legend: a,b,c = different letters indicate differences (P < 0.05) between groups on a specific day; ns= difference not significant; C = control - sausages of usual composition containing nitrites; N+P = sausages produced with nitrites and polyphenols; P = nitrite-free sausages with polyphenols

The results of sensory evaluation are shown in the Figure 6. The colour of P sausages was significantly lower evaluated during the first 30 days (4.4) compared to C and
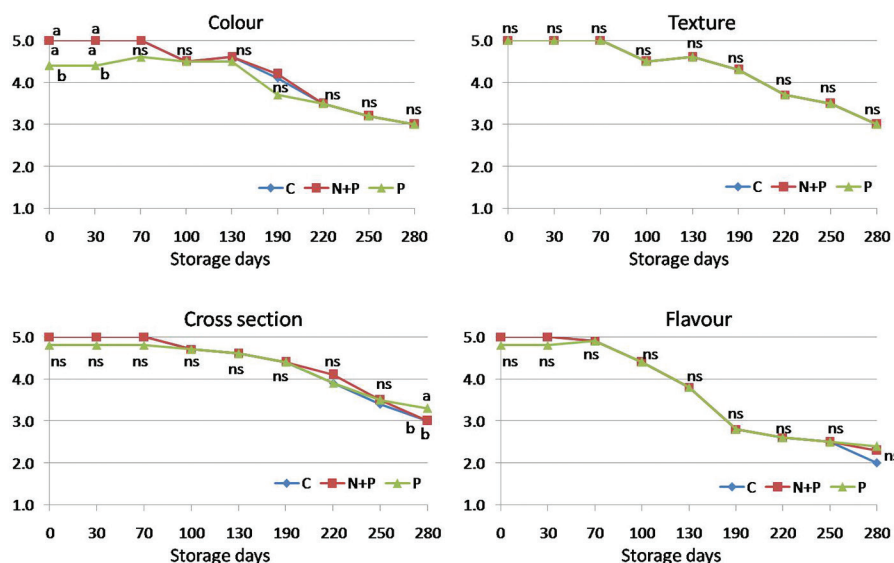
Figure 6. Sensory evaluation of fermented sausages
Legend: a,b,c = different letters indicate differences (P < 0.05) between groups on a specific day; ns= difference not significant; C = control - sausages of usual composition containing nitrites; N+P = sausages produced with nitrites and polyphenols; P = nitrite-free sausages with polyphenols

N+P sausages (5.0), but from day 70 to 130, it received higher ratings (4.5 to 4.6). Concerning other sensory parameters, all products were very similarly rated in all storage phases. The scores for colour, texture and cross section of all experimental groups were above 4.0 till the 190 day of storage, while it decreased to 3.0 till the day 280 of storage. The scores for flavour were above 4.0 till the day 100 while it decreased to 2.0 by control and 2.3 and 2.4 by N+P and P sausages respectively.

Polyphenol contents in sausage extracts

The results of the investigation of polyphenol contents in sausage extracts are shown in Table 2. The most dominant in N+P and P sausages was kaempferol-3-O-glucoside (23.7 and 33.0 ng/g, respectively), followed by quercetin (14.7-15.2 ng/g), luteolin-7-O-glucoside (8.6-12.1 ng/g), catechin (6.8-7.5 ng/g) and syringic acid (5.4-4.0 ng/g). It was observed that the total content of polyphenols detected in the sausage extracts grew during the storage period, raising from 96.9 to 152.2 ng/g in P sausages and from 71.9 to 438.4 ng/g in N+P sausages. The total content of polyphenols detected in sausage extracts was 1.7 (end-product), 2.0 (30th day) and 5.2 (70th day) times higher in N+P sausages compared to P sausages.

Table 2. Polyphenols content change in the sausage extract during production and storage (ng/g)

Polyphenols	stuffing		end-products		storage day 30		storage day 70	
	N+P	P	N+P	P	N+P	P	N+P	P
Gallic acid	0	0	6.7	7.2	8.2	4.5	7.5	3.3
Luteolin	0.4	0.5	4.3	2.6	9.7	5.9	11.6	2.3
Kaempherol	0.4	0.8	13.2	6	18.3	10	21.3	7
Cathehin	6.8	7.5	12.3	6	19.2	6.1	22.5	2
Epicatechin	2	3.1	4.7	2.2	10.1	4.8	15.2	0.8
Quercetin	14.7	15.2	52	28.7	110.5	38	158.2	34.6
Izorhamnetin	0	0	25.6	14.2	30	14.2	35.1	15.2
Kaempherol-3-O-Glc	23.7	33	10.4	7.2	36.8	21.5	50.4	5.8
Isoquercetin/hyperoside	4.5	15	8.9	5.9	32.6	20.6	66.4	4.5
Luteolin-7-O-Glc	8.6	12.1	3.5	2.3	13.6	7.8	18.8	1.7
Ferulic acid	0.1	0.1	0.8	0.1	0.7	1.8	4.1	0.3
Syringic acid	5.4	4	6.4	2.7	6.7	5.5	11.9	3.1
Naringenin	0.8	2.1	2.4	1.7	6.5	4.8	6	1.4
Protocatechuic acid	0	0.3	1.8	1.4	3.2	1.9	3.6	1.5
P-coumaric acid.	3.5	1.9	0.6	0.2	0.9	0.7	1.7	0.5
Vanilic acid	1	1.3	2	1.3	3.1	4.1	4.1	0.9
Σ	71.9	96.9	155.6	89.7	310.1	152.2	438.4	84.9

DISCUSSION

Chemical composition

All the investigated chemical parameters were in the range typical for dry fermented sausages [1,4] and met the regulation requirements [2] concerning moisture and proteins content, as well as the collagen/proteins ratio (Table 1). The nitrite content in the stuffing of the P sausages was significantly lower than in C and N+P sausages as they are produced without nitrite addition. The presence of nitrates in the stuffing of C and N+P sausages could be explained by fast oxidation of nitrites to nitrates after their addition [9]. The detection of nitrates in the stuffing of P sausages could have resulted from their natural presence in spices [32] which were added to sausages during the production. In the end-products, both nitrite and nitrate contents lowered compared to the stuffing in all sausages because of their complex reactions with the sausage matrix compounds [9]. Interestingly, N+P sausages had almost twice as high content of nitrates as did C sausages, which could be attributed to the reactions between phenolic compounds and nitrites that are also observed by other authors [33], but the nature of these reactions in fermented sausage matrices should be further investigated.

Physicochemical and oxidative changes

The recorded a_w value decline (Figure 1) was usual for dry fermented sausages [1] to far lower values than 0.90, which is important for the product safety [33], and remained mainly unchanged during storage. The more intensive a_w value drop was observed in polyphenol-containing sausages (N+P and P), which could be attributed to the simultaneously more intense pH decrease in these sausages (Figure 1). Namely, the decrease in pH value reduces the water-binding capacity of meat, which results in more intense water release and sausage drying [35]. Lower pH values in polyphenol-enriched sausages were also reported by Moawad et al. [36], which was explained by more intense inhibition of spoilage microorganisms that could contribute to higher pH value due to their proteolytic activity. In our study, the highest proteolysis index after the production period was observed in C sausages, which is in accordance with those data. Even more, the microbiological investigation in our study showed significant higher counts of proteolytic bacteria (*Pseudomonadaceae*) in C sausages on days 14 and 21 day of the production period (Figure 3). During storage, the pH value increased simultaneously with proteolysis index (Figure 1), which could be attributed to activity of tissue proteolytic enzymes, as spoilage bacteria were not detected any more, which is normal during the ripening of meat products [28]. In our investigation a higher proteolysis index during storage was observed in polyphenol-enriched sausages but were in range with those typical of fermented sausages [36] and represent the measure of product maturity, which is desirable in dried meat products [28].

After the production period, the acid value which indicates the lipid hydrolysis intensity was higher in polyphenol-enriched sausages, which remained up to day 90 of storage (Figure 2). Afterwards, the highest acid value was observed in the control group. As free fatty acids released via lipid hydrolysis process contribute to the flavour of fermented sausages [6,7,8] these changes could not be considered as undesirable. On the other hand, peroxide and TBARS values, which indicate lipid oxidation, showed different pattern in the first (up to day 130) and second period of storage (from day 130 to day 280). In the first period, the lowest peroxide value and highest TBARS value was observed in P sausages, but as the highest amount of aldehydes in these products was far below the sensory rancidity limit of 2.2 mg MAL/kg [37] and is typical of dry fermented sausages [36], such findings do not represent a significant disadvantage. After 130 days of storage polyphenol-enriched sausages had significantly lower peroxide and TBARS values than C sausages. Additionally, in this period P sausages had significantly lower peroxide and TBARS values then N+P sausages, probably due to the chemical reactions between nitrites and polyphenols [33] which decreased the antioxidant activity of polyphenols in these sausages.

Microbial changes

As the main changes in fermented sausage microbiota occur in the first several weeks [1], microbiological investigation was conducted every 7 days during the production of the sausages (Figure 3), while during the storage time the investigation was carried out on a monthly basis (Figure 4). The results showed that in all sausages the most abundant were lactic acid bacteria (LAB). Although P sausages contained significantly lower counts of LAB on day 14, which could be contributed by a mild antimicrobial effect of polyphenols [37], on other days there was no difference in LAB counts between experimental groups and the counts were all in range of optimum for the fermentation process in sausages [1]. The counts of *Micrococcaceae* was rather similar in all sausages during the production, except on day 7, when N+P sausages contained significantly lower numbers than C and P sausages. *Micrococcaceae* are part of useful microorganisms in fermented sausages which play an important role because of their peroxidase activity and aroma formation [1]. Concerning spoilage bacteria, *Enterobacteriaceae* were not detected after day 14 and *Pseudomonadaceae* after 28 days of production. In P and N+P sausages a significantly lower number of *Pseudomonadaceae* was observed on days 14 and 21, which could be attributed to the antimicrobial effect of polyphenols on *Pseudomonadaceae* already described by other authors [38].

During storage LAB count slightly decreased and was similar in all sausages (Figure 4). Exceptions were observed on days 30 and 190, when LAB counts were significantly lower in polyphenol-containing sausages, which could be the result of the antimicrobial activity of polyphenols [38]. *Micrococcaceae* were not detected after day 220 of storage in all experimental sausage groups. Despite differences between experimental groups, in some phases during storage, the changes in microbiota were in range characteristic of fermented sausages [1]. Concerning pathogen bacteria, *Salmonella* spp. and *Listeria*

monocytogenes were not detected in any of the production and storage phases, which confirms the safety of all sausage groups tested [4].

Colour parameters and sensory evaluation

At the beginning of the storage period, C and P sausages had similar lightness (Figure 5), while N+P sausage was significantly darker. At the same time, C and N+P sausages had similar redness and yellowness while these parameters were significantly lower in P sausages. Such results could be explained by faster red colour formation – the red-purple pigment nitroso-myoglobin is obtained in reactions between nitrite and myoglobin [9]. In fermented sausages produced without additives, like in traditional production, the stable red colour is formed through the slow process of myoglobin reduction and stabilisation [32]. During storage the lightness equalized in all experimental groups on days 130, 250 and 280. Redness was significantly lowered in polyphenol-containing sausages during the whole storage period. Although the N+P sausages contained nitrites, the redness of these sausages was lower than of C sausages, which was probably due to the partial loss of nitrites in reactions with polyphenols [33]. Significantly higher yellowness in C sausages during storage could be attributed to more intense lipid oxidation (see Figure 2) in these, where peroxides and other oxidation products deteriorated red pigments increasing the yellowness [38].

The results of instrumental colour measurements were confirmed by sensory evaluation (Figure 6). The colour of P sausages was significantly lower evaluated during the first 30 days compared to C and N+P sausages, but from day 70 to 130, it received higher ratings because of its more intense red colour, which could be also seen by increased a* value on day 70 observed by instrumental colour measurement (Figure 5). Concerning other sensory parameters, all products were similarly rated in all storage phases. Decreasing marks for all sensory properties, especially after day 130, resulted mostly from oxidative changes which affected primarily the flavour, colour and cross sections of the products. However, polyphenol-containing products were acceptable up to day 280 of storage being evaluated in average about 3.0 for all sensory attributes, while C sausages were rated lowest because of their flavour, which gained score 2.0. Better flavour of polyphenol-enriched sausages could be attributed to the antioxidative role of these compounds [13], which was proved by the lower parameters of lipid oxidation in this study (Figure 2). Additionally, sausages from our experiment were highly evaluated for all examined attributes on day 190 of storage, which is important as dry fermented sausages are usually stored up to 180 days [39]. Thus, this study confirmed the possibility of prolonged storage period of dry fermented sausages, even by nitrite-free sausages enriched with polyphenols.

Polyphenol contents in sausage extracts

The investigation into polyphenol contents in sausage extracts showed that the most dominant was kaempferol-3-O-glucoside (kaempferol-3-O-Glc), followed by quercetin,

luteolin-7-O-glucoside (luteolin-7-O-Glc), catechin, syringic acid and others, being characteristic of grape [40] which was used as a source of polyphenols in our study. During the storage period, it was observed that the content of polyphenols grew, which could be explained by the increase in the concentrations of sausage compounds along with the release of water during drying [1]. Interestingly, the extract of N+P sausages contained at the end of production and 30 days after storage twice, and after 70 days even five times higher total contents of polyphenols as P sausages did, although the same amounts of grape powder were added to their stuffing. This could be explained by the proneness of polyphenols to bind to proteins and build insoluble complexes [41], which caused limited polyphenol extractability from P sausages. Concerning N+P sausages, it should be taken into account that owing to the reactivity of polyphenols with nitrites certain soluble derivates are built [33] which were able to be extracted from the sausages, but it should be confirmed by further studies.

CONCLUSIONS

The addition of polyphenols did not affect the fermentation and drying processes in fermented sausages and products of standard chemical composition were obtained. Nitrite-free sausages contained nitrites in traces. Sausages with both nitrites and polyphenols had higher nitrates contents then the control, which indicates that reactions between polyphenols and nitrites occurred. Polyphenol-enriched sausages had significantly lower peroxide and TBARS values then conventional ones. However, those produced with both nitrites and polyphenols showed higher lipid oxidation than nitrite-free polyphenol-enriched sausages, which indicates lower antioxidative potential in the sausage matrix due to interactions between nitrites and polyphenols. Microbiological processes in all experimental groups were typical of fermented sausages concerning lactic acid bacteria and *Micrococcaceae* counts, and polyphenol-enriched sausages had lower counts of spoilage bacteria (*Pseudomonadaceae*). Although instrumental colour measurement registered differences in lightness, redness and yellowness, the sensory properties of all groups of sausages were highly evaluated during most of the storage period and reached day 280 above the discriminating level. The most dominant polyphenol compound in the sausages was kaemferol-3-O-Glc, followed by quercetin, luteolin-7-O-Glc, catechin and syringic acid.

Nitrite-free polyphenol-enriched sausages reached the same shelf life as conventional sausages, which is a promising result giving hope that polyphenols can be used as potential nitrite substitutes. On the other hand, simultaneous addition of polyphenols and nitrites in fermented sausages is questionable because of indications that interactions between nitrites and polyphenols diminish their positive, especially antioxidative role of both ingredients. Because of the complexity of these reactions, further studies should be conducted to prove their nature in fermented sausage matrices.

Authors' contributions

NA wrote the manuscript, participated in the design of the study, took part in quality, microbiological, sensory and instrumental colour analysis. ÐV participated in the design of the study, analysis and interpretation of data and took part in approval of the final version to be published. PN carried out quality, sensory and instrumental colour analysis and took part in the interpretation of data. SS carried out chemical analysis and took part in the interpretation of data. ÐS performed the statistical analysis and took part in the interpretation of data. BJ carried out microbiological analysis and took part in the interpretation of data. VD participated in the design of the study, drafting the manuscript and revising it critically for important intellectual content and took part in approval of the final version to be published.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DA LI POLIFENOLI MOGU DA SE KORISTE KAO PRIRODNI KONZERVANSI U FERMENTISANIM KOBASICAMA?

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Cilj ovog rada bio je ispitivanje uticaja polifenola na fermentisane kobasice proizvedene sa ili bez dodatka nitrita za vreme skladištenja od 280 dana. Proizvedena su tri tipa kobasica, od kojih su formirane tri eksperimentalne grupe: C - kontrolna grupa – kobasice uobičajenog sastava koje sadrže nitrite, N+P - kobasice sa nitritima i polifenolima i P - kobasice bez nitrita koje sadrže polifenole. Hemijski sastav svih grupa kobasica bio je u opsegu uobičajenom za fermentisane suve kobasice. P kobasice sadržale su 0,3 mg/kg nitrita, dok su C i N+P sadržale 54,8 mg/kg, odnosno 52,2 mg/kg. Kobasice obogaćene polifenolima imale su značajno manji peroksidni i TBARS broj nego C kobasice. Broj mlečno-kiselinskih bakterija dostigao je 8,9-9,9 log cfu/g, ali je u toku skladištenja opao na 4,3-4,8 log cfu/g posle 280dana u svim grupama kobasica. Broj *Micrococcaceae* bio je konstantan (3,5-3,9 log cfu/g). Kod P i N+P kobasica utvrđen je značajno niži broj *Pseudomonadaceae*. C i P kobasice bile su približno jednako svetle (L=50,2,odnosnoL=49,5) dok su N+P kobasice bile tamnije (L=42,5). C i N+P kobasice imale su sličan intenzitet crvne (a*=14,5 odnosno a*=13,2) i žute boje (b*=5,9 odnosno b*=6,4) ali su ove vrednosti bile niže kod P kobasica (a*=8,0 i b*=4,6). Senzorske karakteristike svih proizvoda bile su približno jednako ocenjene. Nešto bolja aroma utvrđena je od kobasica kojima su dodati polifenoli. Najdominantniji polifenol u kobasicama bio je kempferol-3-O-glukozid, a pored njega, bili su utvrđeni kvercetin, luteolin-7-O-glukozid, katehin i siringinska kiselina. Kobasice bez nitrita obogaćene polifenolima postigle su istu održivost kao i uobičajene kobasice koje sadrže nitrite, što predstavlja ohrabrujuće rezultate za mogućnost upotrebe polifenola kao prirodnog konzervansa i zamene za nitrite. Simultana upotreba nitrita i polifenola nije pouzdana zbog interakcija između polifenola i nitrita, što bi trebalo da bude detaljnije istraženo u budućnosti.