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Utilization of brewer's spent grain and mushrooms in fortification of smoked sausages

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Abstract

The aim of this study was to obtain alternative types of smoked sausage by using some vegetable raw materials rich in protein. To this end, smoked sausages with brewer's spent grain [1.5, 3, and 6% w/w], smoked sausages with mushrooms [10, 17, and 30% w/w], and smoked sausages with mixtures of brewer's spent grain-mushrooms [3/8% and 1.5/16% w/w] were prepared. Three of these formulations were selected based on panellist's preference, one for each assortment, and stored under darkness at 4°C and 49% relative humidity for 15 days. Smoked sausage samples were collected at three different storage times (T0, initial state; T1, at day 7 of storage; T2, at day 15 of storage) and evaluated from the physicochemical and microbiological point of view to determine their stability during the intended storage period. Regardless of the type of assortment, storage time had a significant influence on the physicochemical and microbiological properties of smoked sausages. Among all samples, the smoked sausage with a mixture of 3% brewer's spent grain and 8% mushrooms was the most appreciated by the panellists.

Keywords: smoked sausages; brewer's spent grain; button mushrooms; storage time; stability.

Practical Application: Effect of vegetable ingredients addition on smoked sausages composition.

1 Introduction

There is a large variety of industrial and homemade sausages produced and consumed in Romania (Simion et al., 2014). The recipes for smoked sausages vary from one region of the country to another depending on local traditions. Pork meat is usually used as raw material for their preparation but also beef meat, sheep meat, goat meat, or their mixtures.

Considering the positive consumer attitude toward functional food, meat producers must find new ways to satisfy this need; from here and the trend to add vegetable ingredients in meat products for the purpose of nutrient fortification. Previous investigations have shown the effects of wheat protein on smoked poultry sausages (Li et al., 1998); of wheat germ, corn germ, and soy proteins on pork & beef frankfurters (Gnanasambandam & Zayas, 1994); of brewer's spent grain on beef frankfurters (Özvural et al., 2009); and of shiitake mushrooms on pork frankfurters (Pil-Nam et al., 2015).

Brewer's spent grain is a low-cost by-product of brewing, which consists of the fraction remained after the mashing and lautering process. It is rich in dietary fibre and protein because most of the barley starch is removed during mashing (Mussatto et al., 2006; Özvural et al., 2009; Fărcaș et al., 2014; Canedo et al., 2016). The ingestion of brewer's spent grain has proved beneficial in alleviating constipation, accelerating transit time, and reducing total lipids and cholesterol level (Hassona, 1993; Mussatto et al., 2006; Aliyu & Bala, 2011). Therefore, brewer's spent grain can be successfully used in meat products either for their enrichment with dietary fibre or to replace animal protein.

Alongside brewer's spent grain, mushrooms are also rich in protein and dietary fibre (Valverde et al., 2015). Due to their unique and subtle flavour, mushrooms have long been used as food or ingredient in food (Beluhan & Ranogajec, 2011). The most cultivated mushrooms worldwide are white button mushrooms (*Agaricus bisporus*); the substrate for their cultivation is horse manure compost or synthetic compost (Sánchez, 2010; Valverde et al., 2015). This type of mushroom is easy to grow, relatively inexpensive, and easy to find.

The aim of the current work consisted in obtaining new types of smoked sausages with desirable nutritional properties and acceptable sensory characteristics by partial replacement of animal protein with vegetable protein. To the best of our knowledge, this is the first study that describes the utilization of brewer's spent grain and *Agaricus bisporus* mushrooms as meat substitutes in smoked pork sausages. The study was designed to evaluate the influence of storage time (T0, initial state; T1, at day 7 of storage; T2, at day 15 of storage) on physicochemical and microbiological properties of smoked sausages fortified with brewer's spent grain, mushrooms, and a mixture of brewer's spent grain – mushrooms.

2 Materials and methods

2.1 Raw materials and ingredients

The raw material (pork sirloin) used for manufacturing smoked sausages was purchased from a local butchery (Cluj-Napoca, Romania). Brewer's spent grain, the by-product generated

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from the dark lager beer-brewing process, was supplied by the Microbrewery of the Faculty of Food Science and Technology within the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Mushrooms (*Agaricus bisporus*) were purchased from retailers in Transylvania, Romania. The brewer's spent grain was dried in a laboratory oven at 78 °C for 12 h. Its chemical composition consists of 92.1 g/100 g total dry matter, 13.5 g/100 g protein, 4.8 g/100 g fat, and 3.8 g/100 g ash. Button mushrooms were dried at 50 °C for 12 h and milled using a laboratory milling machine. Their chemical composition consists of 94.7 g/100 g total dry matter, 34.7 g/100 g protein, 2.4 g/100 g fat, and 7.2 g/100 g ash. Total dry matter was determined by the oven drying method (Drying Oven, Trade Raypa S.L., Barcelona, Spain), protein content by the Kjeldahl method (Kjeldahl apparatus, J.P. Selecta s.a., Barcelona, Spain), fat content by the Soxhlet method (Solvent extractor, VELP Scientifica, Usmate, Italy), and ash content by the conventional gravimetric method (muffle furnace, Nabertherm GmbH, Bremen, Germany).

2.2 Smoked sausages manufacture

Smoked sausages were produced based on a traditional Romanian recipe at the Meat Pilot Plant of the Faculty of Food Science and Technology (University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania). The control sample [SS, smoked sausages] was prepared from 75.2% pork sirloin, 1.5% salt, 0.3% black pepper, 0.4% paprika, 7.5% garlic, and 15.1% cold water. The meat was cut into pieces and ground then salted and stored in the refrigerator (4 °C) for 24 h. Next, the ground meat was transferred to the meat cutter (Maprotec Maschinen-und Prozeßtechnik GmbH, Germany) for chopping and then mixed with the rest of the ingredients and cold water. The brewer's spent grain and mushrooms were added to the control sample in different proportions (w/w) and thus, eight formulations of smoked sausages were obtained [$SS_{BSG1.5}$, smoked sausages with 1.5% brewer's spent grain; SS_{BSG3}, smoked sausages with 3% brewer's spent grain; SS_{BSG6}, smoked sausages with 6% brewer's spent grain; SS_{M10}, smoked sausages with 10% mushrooms; SS_{M17}, smoked sausages with 17% mushrooms; SS_{M30}, smoked sausages with 30% mushrooms; $SS_{BSG3+M8}$, smoked sausages with 3% brewer's spent grain and 8% mushrooms; $SS_{BSG1.5+M16}$, smoked sausages with 1.5% brewer's spent grain and 16% mushrooms]. The batter was stuffed in 36 mm-diameter pork intestinal casings using a vacuum filling machine (RVF 70, Düker-REX Fleischereimaschinen GmbH, Germany). Sausages were hand-linked at 20-cm intervals, then dried (at 65 °C for 30 min) and smoked (at 75 °C for 1 h) in a smoking chamber (Stawiany, Poland). Further they were pre-cooled at 12 °C for 30 min and cooled at 4 °C in a refrigerating chamber. The smoked sausages thus obtained were kept under refrigeration until analysis.

2.3 Experimental design

To establish the preferred sample for each type of smoked sausages [(i) with brewer's spent grain, (ii) with mushrooms, and (iii) with the mixture of brewer's spent grain-mushrooms], samples were sensory evaluated using the 9-point hedonic test. The three selected samples and the control sample were further stored in a refrigeration room (at 4°C and 49% relative humidity, in darkness), for 15 days to determine their stability during the intended storage period. To this purpose, smoked sausages were sampled initially, at day 7, and at 15 of storage then subjected to physicochemical (protein, fat, moisture, ash, total carbohydrates, and ammonia contents) and microbiological analyses (total viable count (TVC), *Salmonella* species, *Escherichia coli*, and total combined yeasts and moulds count (TYMC)).

2.4 Sensory evaluation of smoked sausages

Sensory characteristics of smoked sausage samples were evaluated by a panel of 30 untrained assessors, with a mean age of 25, selected from students and staff members of the department. Samples were presented in random order in plastic dishes and coded numerically. The 9-point hedonic scale test (1 being "dislike extremely" and 9 being "like extremely") was used to evaluate all smoked sausage samples. Appearance, colour, texture, odour, taste, and overall acceptability were the sensory attributes evaluated.

2.5 Physicochemical evaluation of smoked sausages

Protein, fat, and moisture content were analysed using the Food ScanTM Lab 78810 (Foss Tecator Co., Ltd., Denmark). Two replicates were run for each sample. The results were expressed as g/100 g sample.

Ash content was determined by incineration of the sample in a muffle furnace (L3/11/B170, Nabertherm GmbH, Bremen, Germany). About 3 g of sample was weighed in a porcelain melting pot and maintained at 600 °C for 6 h in the muffle furnace. The following Equation 1 was used to calculate the ash content:

$$(g/100g)\ Ash\ content\ = \frac{W_a}{W_s} \times 100 \quad (1)$$

where: W_a is the weight of ash, in grams; W_s is the weight of sample, in grams.

Total carbohydrates were calculated based on the following Formula 2 from the content of moisture, protein, lipid, and ash (Barros et al., 2008):

$$(g/100g)\ Total\ carbohydrates\ = 100 - (g\ moisture + g\ protein + g\ lipid + g\ ash) \quad (2)$$

Energy value was calculated based on the following Formula 3 from the content of protein, carbohydrate, and lipid using the energy factors (Barros et al., 2007):

$$(kcal/100g)\ Energy\ value\ = 4 \times (g\ protein + g\ carbohydrate) + 9 \times g\ lipid \quad (3)$$

Determination of easily hydrolysable nitrogen content (EHN) was performed based on the method described by SR 9065-7:2007 (International Organization for Standardization, 2007a).

2.6 Microbiological evaluation of smoked sausages

Total viable count (TVC) was determined using the method described in SR EN ISO 4833:2003 standard (International Organization for Standardization, 2003). Detection of

Salmonella spp. and *Escherichia coli* were carried out using the method described in SR EN ISO 6579:2003 + AC:2006 standard and SR EN ISO 16649-2:2007 standard, respectively (International Organization for Standardization, 2006, 2007b). Total combined yeasts and moulds count (TYMC) was determined using the method described in SR ISO 21527-1:2009 standard (International Organization for Standardization, 2009).

2.7 Statistical analysis

To perform statistical tests, Minitab statistical software (version 16.1.0, LEAD Technologies, Inc., Charlotte, NC, USA) was used. The statistically significant differences among smoked sausage formulations were carried out by one-way analysis of variance (ANOVA) at 95% confidence level ($p \leq 0.05$). As a post-test procedure, Tukey's honest significance test was used. Correlations among data were calculated using the Pearson's correlation coefficient.

3 Results and discussion

3.1 Effect of fortification with vegetable ingredients on smoked sausage sensory properties

The eight smoked sausage formulations prepared were subjected to sensory analysis using a 9-point hedonic scale to select the panellists preferred sample for each assortment [(i) smoke sausages with brewer's spent grain, (ii) smoked sausages with mushrooms, and (iii) smoked sausages with the mixture of brewer's spent grain-mushrooms].

The hedonic scores for sensory attributes (appearance, colour, texture, odour, taste, and overall acceptability) of smoked sausage samples are shown in Table 1.

The addition of different levels of brewer's spent grain and a mixture of brewer's spent grain – mushrooms had a significant effect on the overall acceptability of smoked sausages but not a significant one on their appearance, colour, texture, odour, and taste. $SS_{BSG3+M8}$ were the most appreciated by panellists in terms of overall acceptability (8.0 points) and $SS_{BSG1.5}$ the least (6.9 points). The overall score of smoked sausages did not show an increasing or decreasing trend with the level of brewer's spent grain added. Instead, the study of Özvural et al. (2009) showed a decrease of the overall score of fortified beef frankfurters as the content of brewer's spent grain increased. The addition of button mushrooms in smoked sausages up to 30% had no significant effect on their sensory attributes. Instead, the utilization of shiitake mushrooms in the study of Pil-Nam et al. (2015), partly improved the taste and overall acceptability of pork frankfurters.

Analysis of the correlation between physicochemical parameters and sensory properties of smoked sausage revealed a linear relationship ($r^2 = 0.958$; $p = 0.042$) for the pair fat content-texture. It shows that as the fat content increased, by the addition of brewer's spent grain or mushrooms, the appearance of smoked sausages was more appreciated. A similar pattern between fat content and texture was also noticed by Siriken et al. (2009) in Turkish style dry-fermented sausages.

The control sample and samples marked with the highest mean values of overall scores for each assortment (SS_{BSG6}, SS_{M17}, and $SS_{BSG3+M8}$) were further undergone to physicochemical and microbiological evaluation to determine their stability during the 15 days of storage under refrigeration.

3.2 Effects of storage time on smoked sausage physicochemical parameters

The proximate composition, energy value and easily hydrolysable nitrogen content of smoked sausages in different treatments and storage times are presented in Table 2.

Differences in composition and energy value between formulations are due to the type or amount of vegetable ingredients added and storage conditions. Moisture content (Table 2) decreased with storage time from 66.3 to 39.8% (by 1.7 times) in SS, from 53.9 to 35.6% (by 1.5 times) in SS_{BSG6}, from 62.5 to 42.3% (by 1.5 times) in SS_{M17}, and from 55.2 to 35.3% (by 1.6 times) in $SS_{BSG3+M8}$. For this reason, the contents of fat, protein, total carbohydrates, ash, easily hydrolysable nitrogen and energy value increased during storage in each assortment [fat content by 1.4 times in SS, 1.2 times in SS_{BSG6}, 1.5 times in SS_{M17}, and 1.2 times in $SS_{BSG3+M8}$; protein content by 1.4 times in SS, 1.4 times in SS_{BSG6}, 1.6 times in SS_{M17}, and 1.6 times in $SS_{BSG3+M8}$; total carbohydrate content by 2.8 times in SS, 1.5 times

Table 1. Hedonic scores of sensory attributes in smoked sausages.

Sample	Appearance	Colour	Texture	Odour	Taste	Overall acceptability
SS	7.1^{a}	7.7^{a}	6.8^{a}	7.6^{a}	7.4^{a}	7.2^{ab}
$SS_{BSG1.5}$	7.1^{a}	7.4^{a}	6.8^{a}	7.4^{a}	6.9^{a}	6.9^{b}
SS_{BSG3}	6.9^{a}	7.6^{a}	6.7^{a}	7.3^{a}	6.9^{a}	7.2^{ab}
SS_{BSG6}	7.5^{a}	7.9^{a}	6.9^{a}	7.5^{a}	7.3^{a}	7.3^{ab}
SS_{M10}	7.5^{a}	7.9^{a}	7.1^{a}	7.7^{a}	7.6^{a}	7.6^{ab}
SS_{M17}	7.3^{a}	7.8^{a}	7.4^{a}	8.0^{a}	7.7^{a}	7.9^{ab}
SS_{M30}	7.7^{a}	7.8^{a}	7.5^{a}	8.0^{a}	7.9^{a}	7.8^{ab}
$SS_{BSG3+M8}$	8.0^{a}	8.0^{a}	7.5^{a}	8.0^{a}	7.8^{a}	8.0^{a}
$SS_{BSG1.5+M16}$	7.6^{a}	7.7^{a}	7.0^{a}	7.6^{a}	7.4^{a}	7.7^{ab}

Abbreviations: SS, smoked sausages; $SS_{BSG1.5}$, smoked sausages with 1.5% brewer's spent grain; SS_{BSG3}, smoked sausages with 3% brewer's spent grain; SS_{BSG6}, smoked sausages with 6% brewer's spent grain; SS_{M10}, smoked sausages with 10% mushrooms; SS_{M17}, smoked sausages with 17% mushrooms; SS_{M30}, smoked sausages with 30% mushrooms; $SS_{BSG3+M8}$, smoked sausages with 3% brewer's spent grain and 8% mushrooms; $SS_{BSG1.5+M16}$, smoked sausages with 1.5% brewer's spent grain and 16% mushrooms. Different letters in the same column indicate statistically significant differences at $p < 0.05$ (Tukey's test).

Table 2. Physicochemical characteristics and energy value of smoked sausage formulations.

Sample	Storage time	Fat (g/100 g)	Protein (g/100 g)	Moisture (g/100 g)	Ash (g/100 g)	Total carbohydrates (g/100 g)	Energy (kcal/100 g)	EHN (mg NH_3/100 g)
SS	initial state	10.5 ± 0.028^c	11.6 ± 0.042^c	66.3 ± 0.071^a	2.7 ± 0.028^c	9.0 ± 0.113^c	176.8^c	8.5 ± 0.021^c
	7 days	12.5 ± 0.014^b	13.5 ± 0.014^b	54.2 ± 0.071^b	3.5 ± 0.042^b	16.4 ± 0.141^b	231.5^b	18.7 ± 0.141^b
	15 days	14.9 ± 0.035^a	16.3 ± 0.049^a	39.8 ± 0.099^c	4.0 ± 0.042^a	25.1 ± 0.226^a	299.4^a	30.6 ± 0.028^a
		$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***
SS_{BSG6}	initial state	11.4 ±0.064^b	12.9 ± 0.028^c	53.9 ± 0.071^a	2.7 ± 0.014^c	19.1 ± 0.021^c	231.1^c	10.2 ± 0.071^c
	7 days	13.1 ± 0.042^a	15.5 ± 0.057^b	45.4 ± 0.113^b	4.2 ± 0.035^b	21.8 ± 0.134^b	267.4^b	22.1 ± 0.042^b
	15 days	13.3 ± 0.339^a	18.3 ± 0.042^a	35.6 ± 0.099^c	4.6 ± 0.014^a	28.2 ± 0.410^a	305.4^a	27.2 ± 0.127^a
		$p \leq 0.01$**	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***
SS_{M17}	initial state	11.6 ± 0.028^c	14.6 ± 0.028^c	62.5 ± 0.014^a	3.5 ± 0.028^c	7.9 ± 0.071^c	194.4^c	8.5 ± 0.035^c
	7 days	14.9 ± 0.014^b	20.0 ± 0.014^b	52.5 ± 0.028^b	4.2 ± 0.057^b	8.4 ± 0.057^b	247.7^b	17.0 ± 0.127^b
	15 days	17.6 ± 0.141^a	22.8 ± 0.014^a	42.3 ± 0.099^c	5.2 ± 0.071^a	12.1 ± 0.127^a	298.0^a	25.2 ± 0.014^a
		$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***
$SS_{BSG3+M8}$	initial state	13.2 ± 0.014^e	13.8 ± 0.141^c	55.2 ± 0.028^a	3.1 ± 0.014^c	14.7 ± 0.198^c	233.0^c	10.2 ± 0.042^c
	7 days	14.3 ± 0.014^b	18.7 ± 0.028^b	42.3 ± 0.014^b	4.4 ± 0.028^b	20.3 ± 0.085^b	284.5^b	18.7 ± 0.071^b
	15 days	16.0 ± 0.014^a	21.5 ± 0.014^a	35.3 ± 0.014^c	4.7 ± 0.042^a	22.6 ± 0.000^a	320.1^a	28.9 ± 0.085^a
		$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***	$p \leq 0.001$***

Abbreviations: SS, smoked sausages; SS_{BSG6}, smoked sausages with 6% brewer's spent grain; SS_{M17}, smoked sausages with 17% mushrooms; $SS_{BSG3+M8}$, smoked sausages with 3% brewer's spent grain and 8% mushrooms; EHN, easily hydrolysable nitrogen. Values are expressed as mean ± standard deviation of two replicates. Different letters in the same column indicate statistically significant differences at $p < 0.05$ (Tukey's test). Significance: **very significant $p \leq 0.01$. ***extremely significant $p \leq 0.001$.

in SS_{BSG6}, 1.5 times in SS_{M17}, and 1.5 times in $SS_{BSG3+M8}$; ash content by 1.5 times in SS, 1.7 times in SS_{BSG6}, 1.5 times in SS_{M17}, and 1.5 times in $SS_{BSG3+M8}$; energy level by 1.7 times in SS, 1.3 times in SS_{BSG6}, 1.5 in times SS_{M17}, and 1.4 times in $SS_{BSG3+M8}$]. It may be due to the low relative humidity of air from the refrigeration room. At the end of the storage period, the fortified smoked sausages dried out and turned hard. The addition of vegetable ingredients in dry form and high-amount could be other reasons for it next to the moisture loss. Hence, the moisture content in fortified smoked sausages must be equalized by additional cold water.

Easily hydrolysable nitrogen content, an indicator of freshness, also increased with storage time in smoked sausage samples: from 8.5 to 30.6 mg NH_3/100g sample (by 3.6 times) in SS, from 10.2 to 27.2 mg NH_3/100g sample (by 2.7 times) in SS_{BSG6}, from 8.5 to 25.2 mg NH_3/100g sample (by 3.0 times) in SS_{M17}, and from 10.2 to 28.9 mg NH_3/100g sample (by 2.8 times) in $SS_{BSG3+M8}$. According to the Romanian legislation, Order no. 975/1998 (Romania, 1998), the easily hydrolysable nitrogen content in smoked sausages must be less than or equal to 45 mg NH_3/100g sample. Thereby, all smoked sausage samples tested have met this requirement. Özvural et al. (2009) reported an increase of moisture and fat content in beef frankfurters with the amount of added brewer's spent grain and Pil-Nam et al. (2015) an increase of fat content in pork frankfurters with the amount of added shiitake mushrooms.

3.3 Effects of storage time on smoked sausage microbiological parameters

Quantitative detection of TVC, *Salmonella* spp., *E. coli*, and TYMC was performed to establish the contribution of vegetable ingredients microflora to the smoked sausages microbial load and to evaluate their safety. The European regulations on microbiological criteria for meat preparations (European Union, 2005) contain limits for *E. coli* only. The results of smoked sausages microbiological examination are listed in Table 3.

Total viable count detection is a test that quantifies all viable microorganisms that grow aerobically on a food product. Aerobic bacteria are among the factors that cause food spoilage, and their count is often used as an index to reflect the stability of food products at storage (Van Ba et al., 2016). TVC level decreased with storage time (Table 3) from 1.30×10^3 to 5.20×10^2 CFU/g (by 2.5 times) in SS, from 1.45×10^3 to 3.85×10^2 CFU/g (by 3.8 times) in SS_{BSG6}, from 1.56×10^3 to 4.15×10^2 CFU/g (by 3.8 times) in SS_{M17}, and from 1.50×10^3 to 3.13×10^2 CFU/g (by 4.8 times) in $SS_{BSG3+M8}$. *E. coli* level decreased during storage from 3.85×10^2 to 2.50×10^2 CFU/g (by 1.5 times) in SS, from 9.49×10^2 to 5.50×10^2 CFU/g (by 1.7 times) in SS_{BSG6}, from 1.50×10^3 to 7.98×10^2 CFU/g (by 1.9 times) in SSM_{17}, and from 1.99×10^3 to 9.43×10^2 CFU/g (by 2.1 times) in $SS_{BSG3+M8}$. TYMC level also decreased with storage time from 1.25×10^2 to 4.50×10^1 CFU/g (by 2.8 times) in SS, from 4.46×10^2 to 1.58×10^2 CFU/g (by 2.8 times) in SS_{BSG6}, from 2.65×10^2 to 1.47×10^2 CFU/g (by 1.8 times) in SS_{M17}, and from 2.10×10^2 to 1.39×10^2 CFU/g (by 1.5 times) in $SS_{BSG3+M8}$. Bacteria *Salmonella* spp. have not been detected in any smoked sausages sample. The decline of the microbial load (TVC, *E. coli*, and TYMC) during storage is most likely due to the migration of smoke components into sausages in time. It is well-known that smoke components, like short chain fatty acids, aldehydes, and phenols have antimicrobial activity (Ross & Dalgaard, 2004; Cadavez et al., 2016). Opposite to this study, Pil-Nam et al. (2015) reported an increase of the aerobic bacteria level with storage time in unsmoked pork frankfurters

Table 3. Microbiological characteristics of smoked sausage formulations.

Sample	Storage time	TVC (CFU/g)	*Salmonella* spp.	*E. coli* (CFU/g)	TYMC (CFU/g)
SS	initial state	1.30 x 10^3 ± 28.3^a	Not detected in 25 g	3.85 x 10^2 ± 7.1^a	1.25 x 10^2 ± 7.1^a
	7 days	8.50 x 10^2 ± 21.2^b	Not detected in 25 g	3.46 x 10^2 ± 6.4^b	9.50 x 10^1 ± 4.2^b
	15 days	5.20 x 10^2 ± 14.1^c	Not detected in 25 g	2.50 x 10^2 ± 2.8^c	4.50 x 10^1 ± 2.8^c
		$p \leq 0.001$***	-	$p \leq 0.001$***	$p \leq 0.01$**
SS_{BSG6}	initial state	1.45 x 10^3 ± 14.1^a	Not detected in 25 g	9.49 x 10^2 ± 8.5^a	4.46 x 10^2 ± 8.5^a
	7 days	7.55 x 10^2 ± 7.1^b	Not detected in 25 g	6.95 x 10^2 ± 7.1^b	3.01 x 10^2 ± 6.4^b
	15 days	3.85 x 10^2 ± 13.4^c	Not detected in 25 g	5.50 x 10^2 ± 3.5^c	1.58 x 10^2 ± 3.5^c
		$p \leq 0.001$***	-	$p \leq 0.001$***	$p \leq 0.001$***
SS_{M17}	initial state	1.56 x 10^3 ± 21.2^a	Not detected in 25 g	1.50 x 10^3 ± 16.3^a	2.65 x 10^2 ± 4.2^a
	7 days	7.00 x 10^2 ± 14.1^b	Not detected in 25 g	1.19 x 10^3 ± 7.1^b	2.22 x 10^2 ± 2.8^b
	15 days	4.15 x 10^2 ± 7.1^c	Not detected in 25 g	7.98 x 10^2 ± 10.6^c	1.47 x 10^2 ± 1.4^c
		$p \leq 0.001$***	-	$p \leq 0.001$***	$p \leq 0.001$***
$SS_{BSG3+M8}$	initial state	1.50 x 10^3 ± 42.4^a	Not detected in 25 g	1.99 x 10^3 ± 21.2^a	2.10 x 10^2 ± 7.1^a
	7 days	7.30 x 10^2 ± 28.3^b	Not detected in 25 g	1.33 x 10^3 ± 17.7^b	1.93 x 10^2 ± 3.5^a
	15 days	3.13 x 10^2 ± 13.4^c	Not detected in 25 g	9.43 x 10^2 ± 10.6^c	1.39 x 10^2 ± 2.1^b
		$p \leq 0.001$***	-	$p \leq 0.001$***	$p \leq 0.01$**

Abbreviations: SS, smoked sausages; SS_{BSG6}, smoked sausages with 6% brewer's spent grain; SS_{M17}, smoked sausages with 17% mushrooms; $SS_{BSG3+M8}$, smoked sausages with 3% brewer's spent grain and 8% mushrooms; TVC, total viable count; TYMC, total combined yeasts and moulds count. Values are expressed as mean ± standard deviation of two replicates. Different letters in the same column indicate statistically significant differences at $p < 0.05$ (Tukey's test). Significance: **very significant $p \leq 0.01$. ***extremely significant $p \leq 0.001$.

fortified with shiitake mushrooms. This reinforces our above claim on the antimicrobial effect of smoke components and their interaction with food matrix in time.

According to the EC Regulation No 2073/2005 (European Union, 2005), the level of *E. coli* in meat preparations should not exceed 5000 CFU/g. Under the conditions of processing and storage from the present study, all smoked sausage samples have met this requirement.

4 Conclusions

Three types of high-protein smoked sausages were successfully manufactured by the addition of vegetable ingredients (brewer's spent grain, mushrooms, and their mixture). The smoked sausages with 17% mushrooms shown the highest protein content followed by smoked sausages with a mixture of 3% brewer's spent grain and 8% mushrooms, and smoked sausages with 6% brewer's spent grain. Smoked sausage formulations have shown different stability during the period of storage. In general, a loss of moisture content and an increase of ammonia content were noticed in smoked sausages from the physicochemical point of view and a decrease in TVC, *E. coli* and TYMC level from the microbiological point of view. However, smoked sausage samples have met the quality requirement for freshness. Between all formulations evaluated, the smoked sausages sample with the mixture of 3% brewer's spent grain and 8% mushrooms has been most appreciated by the panellists.

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