

Antioxidant activity of mushrooms *in vitro* and in frankfurters

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Abstract: The antioxidant properties of *Boletus edulis*, *Cantharellus cibarius* and *Craterellus cornucopioides* decoctions and the effect of mushroom addition on the total phenolic content and the degree of secondary oxidative changes on lipids in frankfurters was studied. Moderate antioxidant activity was obtained by DPPH assay when mushroom decoctions were tested *in vitro*. Using the conjugated diene method, moderate antioxidant activity was achieved with *Boletus edulis* and *Craterellus cornucopioides* decoction, while with *Cantharellus cibarius* decoction, antioxidant activity was low. Constant amounts of phenolic acid were obtained in frankfurters fortified with *Boletus edulis*, while lipid oxidation on each tested day was several times less than in the control group of frankfurters, throughout two months of refrigerated storage. Generally, these mushrooms could be used as natural antioxidants to interfere with the chemical deterioration of food products and specifically, to extend the shelf life of cooked pork sausages.

Keywords: Antioxidant, Natural extract, Shelf life, Mushroom, Phenolics.

Introduction

Emulsification technology for frankfurter-type sausage production has been used for several hundred years. Frankfurters are the most widespread type of emulsified meat product in the world (Fernández-López *et al.*, 2019). Due to volatility of the used meat, spices and other components, effects of high temperatures in thermal treatment and different storage conditions, cooked sausages are exposed to microbiological (Sachindra *et al.*, 2005), chemical and sensory degradation (Hayes *et al.*, 2011).

Lipid oxidation is recognized as the major problem producing negative effects on the quality and shelf life of meat products, causing oxidative off-flavours, discoloration and spoilage of meat and meat products (Morrissey *et al.*, 1998). Hence, there is presently increasing interest in the control of lipid oxidation in meat products by using antioxidant compounds from synthetic and natural sources (Deda *et al.*, 2007; Özvural & Vural, 2011). However, it is assumed that the existing synthetic antioxidants cause toxicity problems that negatively affect consumers' health, and therefore, usage of these compounds is limited in food (Botterweck *et*

al., 2000). A new trend in partially and totally substituting these synthetic antioxidants with antioxidants from natural sources received the most attention among consumers and meat processors (Ahn *et al.*, 2004; Deda *et al.*, 2007; Yılmaz *et al.*, 2002). Thus, a need for recognizing alternative safe sources of natural antioxidants, specifically of plant origin, has considerably increased in recent years (Skerget *et al.*, 2005).

Mushrooms can be an alternative, less processed and readily available source of natural antioxidants (Đekić *et al.*, 2017a). Drying mushrooms is beneficial, as it concentrates mushroom nutrients such as heat-stable minerals, proteins and umami mixtures. They contain various polyphenolic compounds known as excellent antioxidants (Đekić *et al.*, 2017b) due to their capability of scavenging free radicals by single-electron transfer (Hirano *et al.*, 2001). Therefore, the objective of this study was to investigate the antioxidative effect of *Boletus edulis* (BE), *Cantharellus cibarius* (CaC) and *Craterellus cornucopioides* (CrC) *in vitro* and in frankfurters, in order to examine the potential antioxidant effect of the mushrooms on lipid oxidation during the refrigerated storage of frankfurters.

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Materials and Methods

Sample preparation

In order to obtain a decoction, a mixture (1:10) of dry powdered mushroom and Milli-Q (MQ) water obtained from a Milli-Q water purification system (Merck, Darmstadt) was heated at 80°C, 1 h. The resulting decoction was subjected to all further analyses and also, together with a solid part, it was used as a component in making frankfurters.

Antioxidant capacity of mushroom decoctions *in vitro*

DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay

The method was performed according to *Vunduk et al.* (2015). Extract solutions were prepared in MQ water (Merck, Darmstadt).

Lipid peroxidation

The conjugated diene method according to *Lingnert et al.* (1979) was used.

Preparation of frankfurters

The decoctions of mushrooms were prepared as follows: 60 g of powder was added to 2 L of distilled water and heated at 80°C. The mixture was subjected to reflux for 60 min at 80°C. The decoctions obtained in this way were poured into a plastic container, cooled, and frozen to obtain ice. This was repeated in triplicate and added to frankfurters during production (T1). The same procedure was applied to the following batch (T2), with the exception that 120 g of powder was used in each individual batch. The control frankfurter formulation (C) was prepared with ice obtained from distilled water. All

treatments were formulated to obtain 8 kg batter and each treatment was prepared in triplicate (Table 1).

To determine whether the mushroom decoctions released antioxidant activities in the frankfurters, the common spices such as onion, garlic, etc. were not added, and the sausages were not subjected to smoking, because these spices and smoke can also have antioxidant characteristics which could affect and mask the real effect of the added decoctions in the products. Fresh pork hams (*Musculus Biceps femoris*, *M. Semitendinosus* and *M. Semimembranosus*) and pork back fat at 48 h post-mortem were bought from a local abattoir. All connective tissue and visible fat were removed from the ham muscles. Lean meat and back fat were minced through an 8-mm plate using a meat grinder (Laska 82H, Austria). The meat was transferred to a bowl chopper (Müller EMS, Germany), and salt and polyphosphate were added. The meat was comminuted for 3 min at low speed to extract myofibrillar proteins until the temperature reached 6°C, when other ingredients were slowly added. The temperature of the mixture was not allowed to exceed 12°C (*Costa-Lima et al.*, 2014). After emulsification, meat batters were immediately stuffed into collagen casings (Edicas, Girona, Spain; approximately 22 mm diameter) using a stuffer. The frankfurters were cooked at 80°C in a smokehouse until the core temperature of 72°C was reached. The cooked frankfurters were cooled using a shower. Thereafter, the frankfurters were placed in vacuum bags (3 frankfurters/bag, all from an individual batch) (day 0). The vacuum bags containing frankfurters were then sealed with a tabletop vacuum machine, (MVS 35x, Minipack-Torre SpA, Italy) and stored at 1–4°C. All the experiments were conducted in the pilot meat processing plant at the Animal Source Food Technology Department of the Faculty of Agriculture, University of Belgrade.

Table 1. Formulae of the different types of frankfurters (expressed as % of the different ingredients in the formulae).

	T1 ¹	T2 ¹	C ¹
Meat	48%	48%	48%
Fat	25%	25%	25%
Ice	–	–	25%
Decoction 1	25%	–	–
Decoction 2	–	25%	–
Sodium nitrite	1.7%	1.7%	1.7%
Polyphosphate	0.3%	0.3%	0.3%

Legend: ¹ T1: concentration of 0.75 % mushroom in the batch. T2: concentration of 1.5 % mushroom in the batch. C: control, ice instead of mushroom decoction.

Total phenolic content

Amounts of cooked frankfurters (5 g) were homogenized and extracted with 25 mL of ethanol (96%). The extraction was carried out in an Ultraturax at 15000 rpm for 2 min. The resulting extract was filtered through a 1–2 µm filter paper. After filtration, 1 mL of the extract was added to a cuvette, followed by Folin-Ciocalteu reagent (0.5 mL) and saturated sodium carbonate solution (1 mL). After 1 h, the blue dye formed was measured at a wavelength of 725 nm against a blank using a Jenway 6300 spectrophotometer (Jenway, Felsted, United Kingdom). The phenol content was calculated based on the calibration curve (concentration-dependent absorbance function) of the standard solution of gallic acid. The result was expressed as milligram equivalents of gallic acid per kilogram of the sample – mg GAE kg⁻¹ (Naveena et al., 2013). As a result, the arithmetic means of the phenol content were determined in three finely ground baked frankfurters from each of the sausage batches examined.

TBARs determination

TBARs (2-thiobarbituric acid reactive substances) test was accomplished using the method of Bostoglou et al. (1994), with the following alterations. The total volume of trichloroacetic acid (TCA) was added to the sample, and extraction was performed in an ultrasonic bath XUB 12 (Grant Instruments, Cambridge, UK) (Sojic et al., 2015). A Jenway spectrophotometer 6300 (Jenway, Felsted, United Kingdom) was used to measure absorbances. TBARs analyses were performed on three

frankfurters from each batch, and results were expressed as mean milligrams of malondialdehyde (MDA) per kilogram of frankfurter.

Statistical analysis

The data for phenolic content and TBARs analyses were analyzed using the mixed split plot model ANOVA procedure considering ‘storage day’ and ‘treatment’ as independent variables. Mean differences between groups were tested using Bonferoni’s *post hoc* test operating at a 5% level of significance. All statistical analyses were carried out using SPSS for Windows (SPSS 23.0, Chicago, IL, USA).

The data obtained from the antioxidant analyses of mushroom decoctions *in vitro* were processed using one-way analysis of variance (ANOVA). Tukey’s HSD *post hoc* test was used to distinguish statistical differences between the sausages and storage ($p < 0.05$).

Results and Discussion

Scavenging capacity as measured by DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay

The DPPH assay, based on the measurement of the scavenging capacity of antioxidants towards the stable radical, DPPH, is one of the most common techniques for the determination of antioxidant capacity (Abdullah et al., 2012). Concentration-dependent scavenging activity was observed in all frankfurters (Table 2). Compared to the positive control used (L-ascorbic acid), mushrooms tested

Table 2. Scavenging ability of the mushroom decoctions and the commercial antioxidant, L-ascorbic acid as measured by DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay.

Concentration (mg mL ⁻¹)	BE ¹	CaC ¹	CrC ¹	AA ¹
0.625	21.1±0.95 ^{a,A}	12.72±10.02 ^{ab,A}	5.36±4.38 ^{b,A}	81.33±1.11 ^{c,A}
1.25	35.42±7.13 ^{a,AB}	13.43±2.95 ^{b,A}	16.48±3.85 ^{ab,A,B}	83.65±0.09 ^{c,B}
2.5	32.96±11.56 ^{a,A}	20.57±1.85 ^{ab,A,B}	14.63±6.91 ^{b,A}	83.38±0.15 ^{c,B}
5	43.62±15.97 ^{a,AB}	26.08±9.15 ^{a,B}	24.7±6.02 ^{a,A,B}	84.38±0.26 ^{b,B}
10	57.55±7.19 ^{a,B}	31.2±1.1 ^{b,A}	29.46±1.11 ^{b,B}	81.07±0.49 ^{c,A}
EC ₅₀	4.46	7.41	8.65	–

Legend: ¹Abbreviations: BE – *Boletus edulis*; CaC – *Cantharellus cibarius*; CrC – *Craterellus cornucopioides*; AA – L-ascorbic acid; EC₅₀ value (mg mL⁻¹) is the effective concentration at which DPPH radicals were scavenged by 50%. Notes: Values are mean±standard deviation. Means in the same column with different capital letters and means in the same row with different lowercase letters are significantly different ($p < 0.05$)

by this assay had significantly lower values for each concentration.

The scavenging activity of mushroom extracts towards DPPH free radicals can also be expressed in terms of EC_{50} . The EC_{50} value ($mg\ mL^{-1}$) is the effective concentration at which 50% of the DPPH radicals were scavenged and was obtained by interpolation from the linear regression analysis. EC_{50} values for BE, CaC and CrC were 4.46, 7.41 and 8.65, respectively. A lower EC_{50} value corresponds to a higher antioxidant activity of the mushroom extract. *Puttaraju et al.* (2006) reported EC_{50} values for hot water extract of BE and CaC to be 1.30 and 6.40, respectively.

We wanted to test exactly this method (decoction), due to its convenience (easy, fast, simple and cost-effective) that would mean it could later easily find its way to industrial application. Concerning CrC, the extract used in this study ($8.65\ mg\ mL^{-1}$) had a higher antioxidant effect than the hot aqueous extract of the same mushroom, reported in the study of *Liu et al.* (2012) ($26.37\ mg\ mL^{-1}$). The ability of hot water extract to quench free radicals has been reported by many researchers. *Chirinang and Intarapichet* (2009) reported strong antioxidant activity of mature and baby Ling chih (*Ganoderma tsugae* Murrill), with low EC_{50} of 0.30 and $0.40\ mg\ mL^{-1}$, respectively. The same authors reported moderate antioxidant activity with the same extraction technique for *Pleurotus ostreatus* ($EC_{50} = 11.56\ mg\ mL^{-1}$) and for *P. sajor-caju* ($EC_{50} = 13.38\ mg\ mL^{-1}$). In the study of *Öztürk et al.* (2007), *Agaricus blazei*, *Agrocybe cylindracea* and *B. edulis* displayed moderate DPPH scavenging activities with EC_{50} of 13.75, 26.98 and $15.78\ mg\ mL^{-1}$, respectively. In total, the decoctions of the three mushrooms tested by DPPH

assay showed moderate scavenging ability in comparison to the literature reports on other mushrooms.

Lipid peroxidation as measured by the conjugated diene method

Using the conjugated diene method, at the concentration of $10\ mg\ mL^{-1}$, antioxidant activities of the decoctions were 53.94 ± 5.27 , 22.36 ± 1.34 and $68.75 \pm 0.33\%$ for BE, CaC and CrC, respectively (Table 3). Also, antioxidant activity obtained by these three mushrooms was dose-dependent, reaching their maxima at $10\ mg\ mL^{-1}$. In comparison to the positive control (L-ascorbic acid), our values were significantly lower at each tested concentration.

At the same concentration, measured with the same method, *Mau et al.* (2005) reported antioxidant activity about 60% for Ling chih (*Ganoderma tsugae* Murrill) mushroom. These values correspond to the values obtained in our study for BE and CrC, while the antioxidant activity we obtained from CaC was significantly lower (Table 3). Similar results for antioxidant activity at the same tested concentration for *Pleurotus citrinopileatus* mushroom were reported by *Lee et al.* (2007). In their study, *Tsai et al.* (2007) reported considerably higher values (66.3%, 83.0%, and 85.7% for *Agaricus blazei*, *Agrocybe cylindracea*, and *B. edulis*, respectively) in comparison to our results at the same tested concentration ($5\ mg\ mL^{-1}$).

The conjugated-diene method is based on the ability of the tested compound to slow down the oxidation of conjugated dienes, which can be formed only from polyunsaturated fatty acids (*Huang et al.*, 2005). Since linoleic acid is used as a substrate in

Table 3. Ability of mushroom decoctions and the commercial antioxidant, L-ascorbic acid, to prevent the peroxidation of linoleic acid.

Concentration of active compound ($mg\ mL^{-1}$)	BE ¹	CaC ¹	CrC ¹	AA ¹
0.1	$0 \pm 0^{a,A}$	$0 \pm 0^{a,A}$	$14.14 \pm 2.31^{b,A}$	$78.33 \pm 0.91^{c,A}$
1	$0 \pm 0^{a,A}$	$0 \pm 0^{a,A}$	$27.52 \pm 0.18^{b,B}$	$79.5 \pm 0.75^{c,A,B}$
2.5	$15.63 \pm 1.06^{a,B}$	$0 \pm 0^{b,A}$	$44.07 \pm 1.32^{c,C}$	$79.6 \pm 0.9^{d,A,B}$
5	$40.79 \pm 1.97^{a,C}$	$11.75 \pm 1.98^{b,B}$	$62.5 \pm 1.32^{c,D}$	$80.84 \pm 0.72^{d,B}$
10	$53.94 \pm 5.27^{a,D}$	$22.36 \pm 1.34^{b,C}$	$68.75 \pm 0.33^{c,E}$	$82.73 \pm 0.8^{d,C}$

Legend: ¹Abbreviations: BE – *Boletus edulis*; CaC – *Cantharellus cibarius*; CrC – *Craterellus cornucopioides*; AA – L-ascorbic acid. Notes: Values are mean \pm standard deviation. Means in the same column with different capital letters and means in the same row with different lowercase letters are significantly different ($p < 0.05$)

this assay, it is not completely the same as a biological system and forms only one type of conjugated diene. Hence, this method is not enough for the complete evaluation of the ability of an antioxidant substance to act preventively in complex systems like food. Thus, when testing the antioxidant properties of the final frankfurter products, we also used another method, more specific to meat products.

Total phenolic content of frankfurters during cold storage

The main compounds responsible for the antioxidant activity of mushrooms are phenolics (Elmastas et al., 2007). Our results showed frankfurters fortified with BE mushroom contained the highest amount of phenolics, while CaC and CrC frankfurters contained significantly lower levels of phenolics, on each day of examination during storage (Figure 1). Previously, researchers confirmed the

relationship between total phenolic compounds and antioxidant activity was linear (Annegowda et al., 2013; Isabelle et al., 2010). Also, phenolic compounds in our frankfurters with BE mushroom remained almost unchanged during the storage, which agrees with the findings of Ribas-Agusti et al. (2014) and Van Ba et al. (2016). On the other hand, lower phenolic concentrations throughout the storage period were obtained for frankfurters prepared with extracts obtained from CaC and CrC. Palacios et al. (2011) reported that chlorogenic, *p*-coumaric, homogentisic and protocatechuic acids were found in *B. edulis*, but not in *Cantharellus cibarius* or *Craterellus cornucopioides*. Also, the same researchers reported higher amounts of gallic, gentistic and *p*-hydroxybenzoic acids in BE than in CaC and CrC extracts, which could explain the persistence during storage of the antioxidant compounds in our frankfurters prepared with BE decoction.

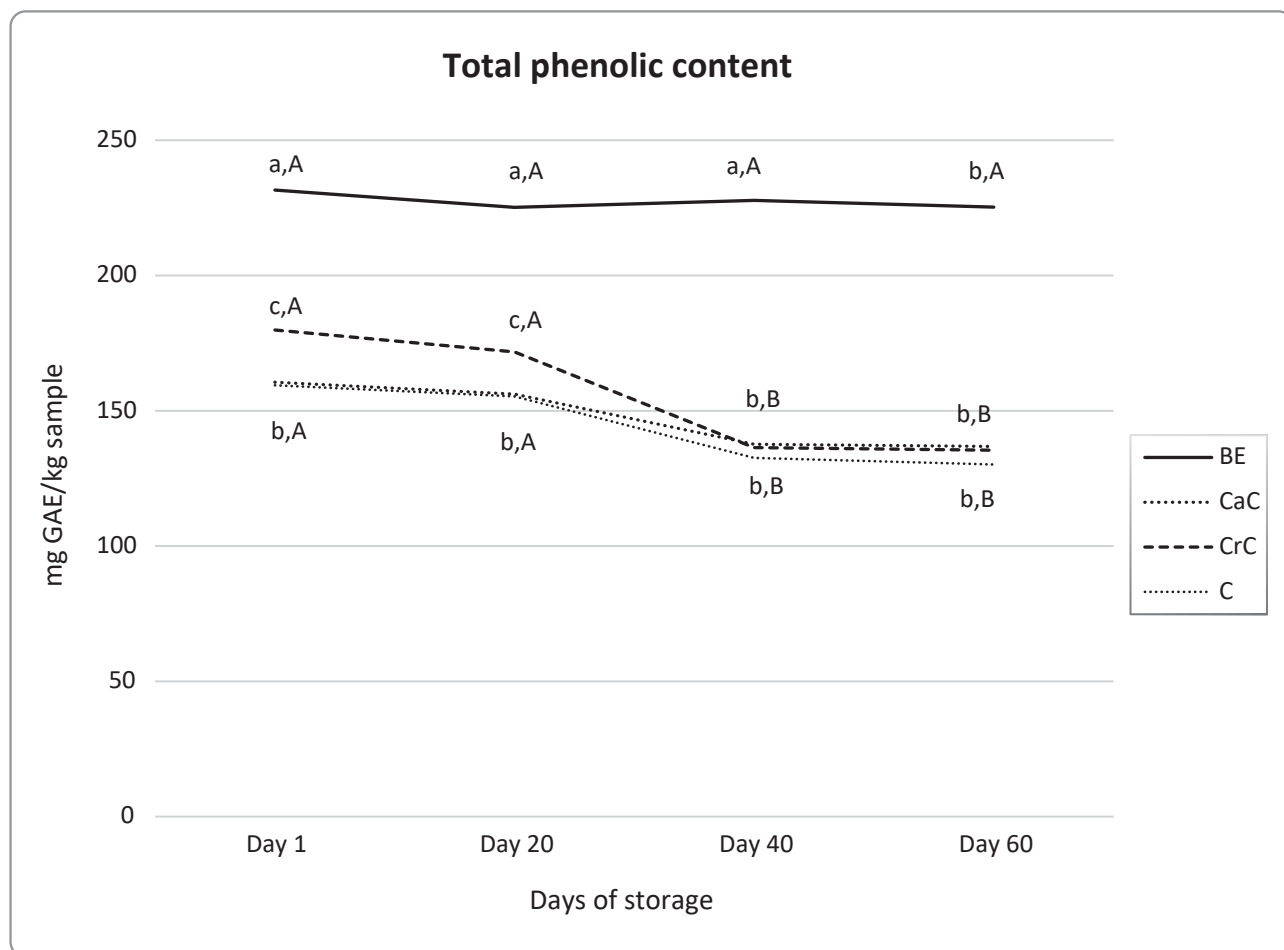


Figure 1. Total phenolic content in frankfurters, expressed as mg GAE/kg of sample.

Legend: ¹Abbreviations: BE – *Boletus edulis*; CaC – *Cantharellus cibarius*; CrC – *Craterellus cornucopioides*; C – control. Values with different lowercase letters (a-c) in the same column (for the different treatments) differ significantly ($p < 0.05$). Values with different uppercase letters (A-B) on the same line (for the same treatment at different storage time) differ significantly ($p < 0.05$).

Table 4. Thiobarbituric acid reactive substances (TBARs) values of frankfurters formulated with different mushroom decoctions during refrigerated storage.

Day of Storage	BE ¹	CaC ¹	CrC ¹	C ¹
Day 1	0.22±0.02 ^{a,A}	0.34±0.04 ^{b,A}	0.14±0.02 ^{c,A}	0.56±0.02 ^{d,A}
Day 10	0.33±0.01 ^{a,B}	0.45±0.01 ^{b,B}	0.31±0.02 ^{a,B}	0.71±0.02 ^{c,B}
Day 20	0.20±0.01 ^{a,A,C}	0.46±0.02 ^{b,B}	0.42±0.03 ^{b,C}	0.59±0.02 ^{c,A,C}
Day 30	0.17±0.01 ^{a,C,D}	0.19±0.01 ^{a,C}	0.17±0.02 ^{a,A}	0.63±0.01 ^{b,C}
Day 40	0.16±0.01 ^{a,D}	0.15±0.01 ^{a,D}	0.11±0.01 ^{a,D}	0.60±0.04 ^{b,C}
Day 50	0.12±0.01 ^{a,E}	0.08±0.01 ^{b,E}	0.09±0.01 ^{ab,D}	0.37±0.01 ^{c,D}
Day 60	0.04±0.02 ^{ab,F}	0.07±0.01 ^{a,E}	0.03±0.01 ^{b,E}	0.16±0.01 ^{c,E}

Legend: ¹Abbreviations: BE – *Boletus edulis*; CaC – *Cantharellus cibarius*; CrC – *Craterellus cornucopioides*; C – control. Notes: Values are mean±standard deviation. Means in the same column with different capital letters (A-F) and means in the same row with different lowercase letters (a-c) are significantly different ($p < 0.05$)

Oxidative changes in frankfurters during cold storage as measured by TBARs assay

The TBARs assay is one of the most frequent methods used for determining the degree of secondary oxidative changes on lipids in meat and meat products (Sojic et al., 2017). On storage day 1, TBARs values for frankfurters with mushrooms were significantly lower in comparison to the control frankfurters (Table 4). This was most likely the consequence of the presence of phenolics in the mushrooms, mixtures that are mainly responsible for the antioxidant activity of many plants (Elmastas et al., 2007). Our TBARs assay results were in accordance with the literature data for a similar type of meat product (Hwang et al., 2015). Additionally, on all days of examination, the incorporation of mushroom decoctions in T1 and T2 frankfurters resulted in significantly lower TBARs values than was found in control frankfurters without mushroom decoction. Relative to the control group, frankfurters with the added mushroom decoctions had up to several times lower TBARs values in some cases. It should be mentioned that all TBARs values were less than 1, the value that Ockerman (1985) found to be the limit for the formation of rancidity in meat products.

Throughout frankfurter storage, trends of increasing and then decreasing TBARs values were noticed. We suggest the decline in TBARs values could be attributed to the creation of MDA, an

intermediate product; until a certain point, the rate of MDA creation was higher than the rate of its disappearance, and thereafter, the reverse was true. Thus, the disappearance rate overshot the rate of creation, and hence, TBARs values declined (Bhattacharya et al., 1988). Similarly, according to Jamora and Rhee (2002), MDA formed during meat product storage might be subjected to intermolecular reactions (polymerization) and reactions with other constituents, especially amino acids/proteins. Since we used mushroom decoction, which is a complex of polysaccharides, proteins, peptides, and free amino acids, it is very likely that polymerization occurred. Therefore, the MDA disappearance (loss) rate during storage can be higher than the rate of formation by lipid oxidation.

Conclusion

The mushroom decoctions used in this study expressed measurable antioxidative effects in the prepared frankfurters. We believe mushroom decoctions show potential and should be considered as a natural replacement of the commercial antioxidants in this kind of meat product. Further research is needed to evaluate the overall quality of frankfurters with added mushroom decoction, to be sure that mushroom addition does not have a detrimental effect on the quality and safety parameters of the final product.

Antioksidativna aktivnost gljiva in vitro i u frankfurterima

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A p s t r a k t: Ispitivane su antioksidativne karakteristike dekokta vrganja (*Boletus edulis*), lisičarke (*Cantharellus cibarius*) i crne trube (*Craterellus cornucupiooides*) i uticaj dodatka gljiva na ukupni sadržaj fenolnih komponenti i stepen sekundarnih oksidativnih promena na mastima u frankfurterima. Umerena antioksidativna aktivnost dobijena je korišćenjem DPPH metode prilikom testiranja dekokta gljiva in vitro. Korišćenjem konjugen dienske metode, umerena antioksidativna aktivnost postignuta je sa dekoktima vrganja (*Boletus edulis*) i crne trube (*Craterellus cornucupiooides*), dok je sa lisičarkom (*Cantharellus cibarius*) taj efekat bio slab. Konstantne količine fenolnih kiselina dobijene su u frankfurterima sa dodatkom vrganja (*Boletus edulis*), dok je oksidacija lipida prilikom svakog testiranog dana bila nekoliko puta manja u poređenju sa kontrolnom grupom frankfurtera, tokom dva meseca skladištenja u frižideru. Generalno, ove gljive mogu biti korišćene kao prirodni antioksidansi kako bi ometali hemijske produkte kvara i produžili rok trajanja kuvanih svinjskih kobasica.

Ključne reči: Antioksidanti, prirodni ekstrakti, održivost, vrganji, fenoli.

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