Chemical characterization of *Agaricus bohusii*, antioxidant potential and antifungal preserving properties when incorporated in cream cheese

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Abstract

Agaricus bohusii Bon is an edible and prized mushroom especially common in Serbia and southern Europe. Herein, this species was chemically characterized by evaluation of nutritional value (e.g. macromolecules, free sugars and fatty acids), bioactive compounds (e.g. tocopherols, phenolic compounds and organic acids), and antioxidant activity of its methanolic extract (e.g. scavenging activity, reducing power and inhibition of lipid peroxidation). Its antifungal preserving properties were also evaluated after incorporation of *A. bohusii* extract in cream cheese, using fungus *Penicillium verucosum* var. *cyclopium* as source contaminant. Comparison of sensory evaluation of cream cheese alone and enriched with *A. bohusii* extract was recorded. According to our findings, *A. bohusii* was rich source of carbohydrates and proteins, containing γ-tocopherol as the only isoform of tocopherols. Polyunsaturated fatty acids also predominated over mono and unsaturated fatty acids. *p*-Hydroxybenzoic and *p*-coumaric acids were the phenolic acids identified in the studied sample; two related compounds were found in higher amounts: γ-L-glutaminyl-4-hydroxybenzene and cinnamic acid. Malic, oxalic and fumaric acids were the organic acids identified and quantified in *A. bohusii*. High concentration of total phenolics was in correlation with strong antioxidant capacity. Methanol extract successfully inhibited development of *P. verucosum* var. *cyclopium* in cream cheese, tested at room temperature after 7 days of inoculation. Sensory evaluation showed cream cheese in combination with *A. bohusii* extract slightly more acceptable to panelists than cream cheese alone.

Keywords: Agaricus bohusii; Chemical characterization; Antioxidant activity; Enriched cream cheese; Antifungal preservation; Sensory evaluation.

1. Introduction

Mushrooms are widely appreciated all over the world for their nutritional properties (Kalač, 2009), and also for their pharmacological value (Ferreira et al., 2009 and 2010). They have been considered valuable health foods being a source of many different nutraceuticals such as unsaturated fatty acids, phenolic compounds or tocopherols (Barros et al., 2009; Heleno et al., 2010; Kavishree et al., 2008). Furthermore, mushrooms can play an important role helping the endogenous defense system in the maintenance of equilibrium between free radicals production and antioxidant defenses in the organism (Ferreira et al., 2009). Antimicrobial properties of mushrooms have also been described but using mostly agar diffusion method, and not microdilution method to verify the findings, despite microdilution method gives results more similar to those of clinical findings (Kalemba & Krunicka, 2003). There are just a few studies testing antimicrobial properties of mushrooms by microdilution method, showing mushrooms as rich sources of antimicrobials (Šiljegović, 2011).

Agaricus bohusii Bon is an edible and prized mushroom especially common in Serbia and southern Europe. It could be found single or in caespitose under broadleaved trees. In summer, it generally appears after showers or it arises in early autumn, occurring mainly in alluvial forests and on synanthropic habitats (Kreisel, 2006). *A. bohusii* is a large mushroom; its fruiting body includes a cap with 20-30 cm and a stem with 25×3 cm. It has prominent and very pointed radiating cap scales on a pale ground (Kibby, 2007), (**Figure 1**). In Hungary, it is considered a care-demanding species, being part of the "Red Data List of Macrofungi in Hungary" (Siller and Vasas, 1995).

A. bohusii can be found in several regions of Serbia including: Belgrade area, Lazarevac, Mladenovac and Smederevska Palanka. This species is not being processed

in Serbian literature. Presumably, it is a rare species, but it is also possible that *A. bohusii* is misidentified as it has very similar features with *A. augustus* and *A. langei* (Davidović, 2002).

Several studies on mushrooms have been made in Balkan regarding species from this region (Harhaji et al., 2008; Leskosek-Cukalovic et al., 2010; Potočnik et al., 2010). However, as far as we know, there are no studies about the species *A. bohusii* neither from Serbia nor from any other country.

In the present study, a detailed chemical characterization of *A. bohusii* was performed, including evaluation of nutritional value (e.g. macromolecules, free sugars and fatty acids), bioactive compounds (e.g. tocopherols, phenolic compounds and organic acids), and antioxidant activity of its methanolic extract (e.g. scavenging activity, reducing power and inhibition of lipid peroxidation). We further analyzed antifungal preserving properties of the mentioned *A. bohusii* extract incorporated in cream cheese, using fungus *Penicillium verucosum* var. *cyclopium* as source contaminant. Comparison of sensory evaluation of cream cheese alone and enriched with *A. bohusii* extract was recorded.

2. Material and Methods

2.1. Mushroom species

Agaricus bohusii Bon was collected during July of 2011 in Jabučki rid, Northern Serbia and authenticated by Dr. Jasmina Glamočlija (Institute for Biological Research). A voucher specimen has been deposited at the Fungal Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, under number Ab-JGMSDS-2011.

2.2. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, sugars (D(-)-fructose, D(-)-mannitol, D(+)-raffinosepentahydrate, and D(+)-trehalose) and tocopherols (α-, β-, γ-, and δ-isoforms) standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards (γ-L-glutaminyl-4-hydroxybenzene, *p*-hydroxybenzoic acid, *trans*-*p*-coumaric acid, and cinnamic acids) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Methanol and all other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Mueller–Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Phosphate buffered saline (PBS) was obtained from Sigma Chemical Co. (St. Louis, USA).

2.3. Chemical characterization of Agaricus bohusii

2.3.1. Nutritional value. The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content (N×4.38) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by

difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Sugars composition. Free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Reis et al., 2011). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.3. Tocopherols composition. Tocopherols were determined following a procedure previously optimized and described by the authors (Heleno et al., 2010). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in µg per 100 g of dry weight.

2.3.4. Fatty acids composition. Fatty acids were determined after a transesterification procedure as described previously by the authors (Reis et al., 2011), using a gas

chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (GC-FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7). The results were expressed in relative percentage of each fatty acid.

2.3.5. Phenolic compounds composition. Phenolic acids were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by Barros et al. 2009. Detection was carried out in a diode array detector (DAD) using 280 nm as the preferred wavelength. The phenolic compounds were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in µg per g of dry weight.

2.3.6. Organic acids composition. Samples (~2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), the sample was filtered through 0.2 µm nylon filters. The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Coperation). Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 µm, 250 mm × 4.6 mm i.d) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at

215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of dry weight.

2.4. Preparation of the extract

Samples (~15 g) were extracted by stirring with 400 mL of methanol (25ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 200 mL of methanol (25ºC at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40ºC (rotary evaporator Büchi R-210) to dryness. The extract was redissolved in i) methanol for antioxidant activity assays or ii) sterilized distillated water containing 0.02% Tween 80 for antimicrobial activity assays.

2.5. Evaluation of antioxidant potential of Agaricus bohusii extract

2.5.1. General. Successive dilutions were made from the stock solution and submitted to *in vitro* assays already described by the authors Reis et al. (2011) to evaluate the antioxidant activity of the samples. The sample concentrations (mg/mL) providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as positive control.

2.5.2. Folin-Ciocalteu assay. One of the extract solutions (0.625 mg/mL; 1 mL) was mixed with *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany). Gallic acid was used to obtain

the standard curve and the reduction of *Folin-Ciocalteu* reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.5.3. Reducing power or ferricyanide/Prussian blue assay. The extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA).

2.5.4. DPPH radical-scavenging activity. This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on 96 wells plate consisted of a solution by well of the extract solutions with different concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: $\% \text{ RSA} = [(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

2.5.5. Inhibition of β-carotene bleaching or β-carotene/linoleate assay. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40ºC under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400

mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: Absorbance after 2h of assay/initial absorbance) × 100.

2.5.6. Thiobarbituric acid reactive substances (TBARS) assay. Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for10 min. An aliquot (100 μL) of the supernatant was incubated with the different concentrations of the samples solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.6. Evaluation of antifungal preserving properties of Agaricus bohusii extract in cream cheese

2.6.1. Cream cheese. Full-fat cream cheese "a la Kajmak", produced from cow milk by Mlekara Šabac was purchased from a local supermarket and kept in refrigerator at 4°C utill further analyzes. All the samples were used before expiring date of the product. Composition of the cream cheese stated on the packaging was: energy, 242 kcal; fat, 23.5 g; proteins, 6.1 g and lactose, 1.1 g, all values expressed by 100 g. The packaging showed the presence of no artificial preservatives. Experiments of inoculating Malt Agar (MA) and Muller–Hinton Agar (MHA) plates with cheese dilluted 1 in 10 with phosphate buffered saline (PBS) and kept at 25°C and 37°C, for 48 hours, showed no bacterial nor fungal contamination of the product.

2.6.2. Isolation and identification of cream cheese contaminant fungus. Cream cheese was left open at 4°C for 3 days. After that period we repeated the experiment with dillutions to check for the contaminants. MA plates with penicillin were inoculated with different dillutions of cream cheese in PBS and kept at 25°C in order to investigate possible fungal contaminants. Pure fungal culture was isolated from inoculated MA plates after 48 hours and identified by Dr. Milica Ljaljević-Grbić (Faculty of Biology, University of Belgrade) as *Penicillium verucosum* var. *cyclopium* and deposited to Micromycetes Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, under number Pvc-DS-11.

2.6.3. In vivo antifungal assay in cream cheese. For the analyzes we used modified method that was previously described by Back et al. (1993); 10 g of cream cheese were

added to 90 mL of PBS in sterile flasks and homogenized for 2 min. *Agaricus bohusii* extract solution (*vide* section 2.4.) was added to the cheese mixture to achieve final concentrations of 10, 20, 40, 60, 80 and 100 mg/mL. The controls contained PBS but not mushroom extract. The flasks were homogenized for 30 s to ensure mixing of the mushroom extract with the cream cheese dilution.

The cheese mixture was inoculated with ~10^4 CFU of *Penicillium verucosum* var. *cyclopium* that had been prepared by growing fungus at 25°C for 10 days on MA plates. Spore suspension was adjusted with sterile saline to approximately 1×10^4 CFU per mL of cheese. The inoculum was mixed thoroughly with the cheese mixture by hands and and the concentration of *P. verucosum* var. *cyclopium* was determined in the cheese on 0 hour, 2^{nd}, 4^{th} and 7^{th} day using the serial dilution and spread plate technique (Jay, 1992). Experimental flasks were divided in two groups: one group was kept at 25°C and the other at 4°C. Both groups contained equal amount of *A. bohusii* extract. PBS was used as the diluent and fungus was cultured on solid MA plates at 25°C for 24h for samples kept at room temperature, and at 25°C for 48h for samples kept at 4°C. The fungus grown in samples at lower temperature (4°C) needed more time to adapt to temperature and to form colonies. Prior to removing samples, the contents of the flasks were mixed by manual gently squeezing. The results were expressed as inhibition percentage of original inoculum.

2.6.4. Sensory evaluation. Sensory evaluation of cream cheese and cream cheese enriched with *A. bohusii* extract was assessed by a group of 10 untrained panelists. Panelists were selected among students and staff of the Department of Plant Physiology (Mycological Laboratory). Cream cheese samples enriched with *A. bohusii* extract were prepared by adding the highest amount used in previous experiments (100 mg to 1 mL

of cream cheese). The panelists were asked to evaluate overall acceptance of the food samples (cream cheese alone and cream cheese enriched with mushroom extract) on a scale from 5 to 1; indicating decreasing taste. A general taste score was calculated as the average of all grades. Overall acceptance was evaluated using a 5-point scale, according to a previous report (Sorheim et al., 1996), where 1 = extremely dislike, 2 = dislike, 3 = neither like nor dislike, 4 = like; 5 = extremely like. Results were expressed as average grades given by 10 panelists.

2.7. Statistical analysis

Three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD), and analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 18.0 program.

3. Results and Discussion

The results of the chemical characterization of *Agaricus bohusii* are shown in Table 1. Carbohydrates were the most abundant macronutrients (69.79 g/100 g dw), followed by proteins (18.06 g/100 g dw). Ash and fat contents were low (9.04 g/100 g dw and 3.12 g/100 g dw, respectively). The energetic contribution of *A. bohusii* sample was 379.44 kcal/100 g dw. Data obtained is in agreement with the stated by different authors who reported mushrooms as good sources of carbohydrates and proteins as well as poor in fat and calories (Kavishree et al., 2008; Kalač, 2009; Ouzouni et al., 2009; Reis et al., 2011). This way, *A. bohusii* could also be an equilibrated valuable food since it maintains these parameters. The main sugars found in this species were mannitol (13.01 g/100g dw) and trehalose (0.47 g/100g dw). Mannitol is the most abundant polyol in the

sporocarps of Basidiomycetes and Ascomycetes (former classification) (Lewis and Smith, 1967) and is an energy store as well as an osmoticum (Hammond and Nichols, 1976). Because of their mannitol contents, mushrooms are useful for diabetic foods (Hamano, 1997). Trehalose is a common sugar component of most immature sporocarps and it may function as a reserve which is metabolized when the sporocarps are maturing (Koide et al., 2000). Thus, these two molecules are typically found in several species of mushrooms (Kalač, 2009; Reis et al., 2011). Regarding tocopherols, only γ-tocopherol was found in the studied sample (27.82 µg/100g dw). Nevertheless, the presence of other isoforms in mushrooms was already described (Jayakumar et al., 2009; Heleno et al., 2010). Concerning the fatty acids composition of *A. bohusii* (**Table 2**), polyunsaturated fatty acids (PUFA, 73.79% of total FA) predominated over saturated fatty acids (SFA, 21.05% of total FA) and monounsaturated fatty acids (MUFA, 5.17% of total FA). Edible mushrooms are considered a good source of many different nutraceuticals such as unsaturated fatty acids (Yilmaz et al., 2006; Kavishree et al., 2008), and *A. bohusii* is not an exception. The FA determined in higher percentages, were linoleic acid (C18:2n6; 72.85% of total FA); palmitic acid (C16:0; 10.10% of total FA) and oleic acid (C18:1n9; 4.50% of total FA). Other authors also found these FA in higher amounts in several mushrooms.

Analyzing the results obtained for antioxidant activity, *A. bohusii* revealed a high concentration of total phenolics (89.59 mg GAE/g extract), indicating a high quantity of molecules with reducing capacity. The EC_{50} values obtained for all the assays used to evaluate reducing power, free radical scavenging activity and lipid peroxidation inhibition were low (≤ 1.29 mg/mL), indicating a high antioxidant potential of the studied species.

Two phenolic acids, *p*-hydroxibenzoic and *p*-coumaric acids, and two related compounds, γ-L-glutaminyl-4-hydroxybenzene (GHB) and cinnamic acid, were found in the sample (**Table 3**). GHB (32.44 µg/g dw) and *p*-hydroxibenzoic acid (9.21 µg/g dw) were the most abundant compounds.

Among organic acids, it was possible to quantify malic (43.89 mg/g dw), oxalic (12.97 mg/g dw) and fumaric (8.31 mg/g dw) acids. These organic acids play an important role in human body being part of the Krebs cycle. Malic acid is a member of C4-dicarboxylic acid family and an intermediate of the tricarboxylic acid (TCA) cycle. This organic acid has been employed in food additives and in polymer and pharmaceutical industries. During a process of pathogenesis by some fungi, oxalic acid is produced while their mycelium is in advancement, in order to make the plant more susceptible (Nagarajkumar et al., 2005). Thus, the production of this organic acid by fungi could have toxicity effects. Fumaric acid, an unsaturated dicarboxylic acid, possesses interesting biological effects such as anti-inflammatory, neuroprotective and chemopreventive activity. For example, it could be used in some therapies for patients with severe *psoriasis vulgaris*. It acts also as an antimicrobial agent for fruits and vegetables preservation (Altmeyer et al., 1994; Baati et al., 2011).

According to recent reports by Perven et al. (2011) species of *Penicillium, Aspergillus, Cladosporium* and unidentified actinomycetes were isolated from cream cheese and their distribution and frequency of occurrence were higher in samples kept at room temperature than for samples kept in refrigerator. We isolated only one contaminant species *P. verucosum* var. *cyclopium*; we kept sample of cream cheese opened for a short period of time. It is undisputed that more fungal contaminants may occur in cream cheese, but we primarily investigated possible antimicrobial preservation of cream

cheese with *A. bohusii* extract towards the fastest fungal contaminant able to develop on the dairy product tested.

Results of *in vivo* cream cheese preservation by *A. bohusii* methanolic extract are presented in **Table 4** and **Figure 2**. From the enclosed table it is evident that different concentrations had different effect on *P. verucosum* var. *cyclopium* growth in cream cheese.

All the concentrations of extract tested in cream cheese kept at room temperature of 25°C for 7 days had the same effect, 100% inhibition of *P. verucosum* var. *cyclopium* spore development in cheese. After that period there were no spore cells of the contaminant tested that retained natural ability of germination. Growth inhibition percentage was recorded on the 2nd day from the beginning of observation. During the period of storage a gradual inhibition of *P. verucosum* var. *cyclopium* growth was achieved in all concentrations used. With exception of 60 mg/mL and 80 mg/mL of examined extract in cream cheese, the growth of food-poisoning fungus was inhibited 100% at the 4th day of experiment.

As for the samples kept at 4°C for 7 days, we recorded slightly different results. Growth inhibition percentage was lower than for the samples kept at 25°C. No growth inhibition was noticeable neither at 0h, nor at the 2nd day of experiment. In that period spore cells of *P. verucosum* var. *cyclopium,* prepared by dilution of cream cheese samples, were viable and able to produce mycelium on MA plates. We recorded growth inhibition for concentrations used 10 mg/mL, 20 mg/mL, 40 mg/mL and 60 mg/mL at the 4th day of cream cheese storage at 4°C. Growth inhibition percentage was in range from 13.3% for 40 mg/mL to 83.3% for concentration of *A. bohusii* extract of 60 mg/mL. Inhibition was not accomplished with 80 mg/mL and 100 mg/mL after four days from the beginning of inoculation. Noticeable growth inhibition percentage was observed for all tested

samples and was in range 80.0% - 90.0%, at the 7th day of experiment. Observation of growth inhibition was aborted after 7 days, because spore cells of *P. verucosum* var. *cyclopium* started to germinate in cream cheese medium, to produce mycelium and biomass of the contaminant increased.

Our study of antimicrobial preservation is supported by previous findings of antimicrobial properties of different mushroom species belonging to the genus *Agaricus*. Antimicrobial action of *A. brasiliensis* ethanol extracts was revealed (Lund et al., 2009). Investigation of antimicrobial activity of three *Agaricus* species was published recently by Ozturk et al. (2011). They presented antimicrobial effect of methanol extract of Agaricus species against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and two species of yeast. Methanol extracts from both *A. bisporus* and *A. essettei* did not show any antibacterial activity against Gram-negative bacteria at test concentration, Gram-positive bacteria were inhibited by these extracts. But, only *A. bitorquis* extract has some effects against three of Gram-negative bacteria namely *Y. enterecolitica* RSKK 1501, *K. pneumoniae* ATCC 27736 and *P. vulgaris* RSKK 96026.

Sensory evaluation of cream cheese alone and cream cheese enriched with *A. bohusii* methanol extract is presented in **Table 5**. Average grade for cream cheese alone was 4.1 indicating the panelist liked the product; average grade for cream cheese enriched with *A. bohusii* methanol extract was 4.8 indicating that the same panelist gave slight priority to the enriches product. Sensory analyses of different types of cream cheeses have been done in the past and the work was published by Brighenti et al. (2008).

Overall, *A. bohusii* was rich source of carbohydrates and proteins, containing γ-tocopherol as the only isoform of tocopherols. Polyunsaturated fatty acids also

predominated over mono and unsaturated fatty acids. *p*-Hydroxybenzoic and *p*-coumaric acids were the phenolic acids identified in the studied sample; two related compounds were found in higher amounts: γ-L-glutaminyl-4-hydroxybenzene and cinnamic acid. Malic, oxalic and fumaric acids were the organic acids identified and quantified in *A. bohusii*. High concentration of total phenolics was in correlation with strong antioxidant capacity. Methanol extract successfully inhibited development of *P. verucosum* var. *cyclopium* in cream cheese, tested at room temperature after 7 days of inoculation. Sensory evaluation showed cream cheese in combination with *A. bohusii* extract slightly more acceptable to panelists than cream cheese alone. Previous studies reported enhanced antioxidant activity of tocopherols with some phenolic compounds in cream cheese (Becker et al., 2008). According to our *in vitro* antioxidant studies, mushroom investigated in this work is a very good source of antioxidants and might also be used for antioxidant preservation of cream cheese.

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Table 1. Nutritional value, sugars and tocopherols composition of *Agaricus bohusii* (mean ± SD).

Nutritional value		**Individual compounds**	
Ash (g/100g dw)	9.04 ± 0.01	**Sugars**	
Proteins (g/100g dw)	18.06 ± 0.46	Mannitol (g/100g dw)	13.01 ± 0.85
Fat (g/100g dw)	3.12 ± 0.02	Trehalose (g/100g dw)	0.47 ± 0.03
Carbohydrates (g/100g dw)	69.79 ± 0.47	**Tocopherols**	
Energy (kcal/100g dw)	379.44 ± 0.10	γ-tocopherol (μg/100g dw)	27.82 ± 0.93

dw – dry weight

Table 2. Fatty acids composition of *Agaricus bohusii* (mean ± SD).

Fatty acids			
C6:0	0.04 ± 0.00	C18:3n3	0.25 ± 0.01
C8:0	0.01 ± 0.00	C20:0	2.46 ± 0.02
C10:0	0.01 ± 0.00	C20:1	0.15 ± 0.01
C12:0	0.07 ± 0.00	C20:2	0.15 ± 0.00
C13:0	0.01 ± 0.00	C20:3n3+C21:0	0.47 ± 0.01
C14:0	0.31 ± 0.00	C20:5n3	0.07 ± 0.00
C15:0	0.65 ± 0.01	C22:0	2.12 ± 0.08
C16:0	10.10 ± 0.03	C22:1n9	0.04 ± 0.00
C16:1	0.48 ± 0.00	C23:0	0.33 ± 0.01
C17:0	0.69 ± 0.01	C24:0	1.30 ± 0.08
C18:0	2.96 ± 0.00	Total SFA (% of total FA)	21.05 ± 0.24
C18:1n9c	4.50 ± 0.00	Total MUFA (% of total FA)	5.17 ± 0.01
C18:2n6c	72.85 ± 0.24	Total PUFA (% of total FA)	73.79 ± 0.22

C6:0 (Caproic acid); 2- C8:0 (Caprylic acid); C10:0 (Capric acid); C12:0 (Lauric acid); C14:0 (Myristic acid); C15:0 (Pentadecanoic acid); C16:0 (Palmitic acid); C16:1 (Palmitoleic acid); C17:0 (Heptadecanoic acid); C18:0 (Stearic acid); C18:1n9c (Oleic acid); C18:2n6c (Linoleic acid); C18:3n3 (Linolenic acid); C20:0 (Arachidic acid); C20:1 (cis-11-Eicosenoic acid); C20:2 (cis-11,14-Eicosadienoic acid); C20:3n3+C21:0 (cis-11,14,17-Eicosatrienoic acid and Heneicosanoic acid); C20:5n3 (cis-5,8,11,14,17-Eicosapentaenoic acid); C22:0 (Behenic acid); C22:1n9 (Erucic acid); C23:0 (Tricosanoic acid); C24:0 (Lignoceric acid).

Table 3. Antioxidant activity, phenolic compounds and organic acids of *Agaricus bohusii* extract (mean ± SD).

Antioxidant activity		**Phenolic and related compounds**		**Organic acids**	
Total Phenolics (mg GAE/g extract)	89.59 ± 3.07	γ-L-glutaminyl-4-hydroxybenzene (μg/g dw)	32.44 ± 0.81	Oxalic acid (mg/g dw)	12.97 ± 0.56
Reducing power (EC_{50}; mg/mL)	1.29 ± 0.01	*p*-Hydroxybenzoic acid (μg/g dw)	9.21 ± 0.21	Malic acid (mg/g dw)	43.89 ± 1.01
DPPH radical-scavenging activity (EC_{50}; mg/mL)	1.03 ± 0.04	*p*-Coumaric acid (μg/g dw)	1.28 ± 0.13	Fumaric acid (mg/g dw)	8.31 ± 0.06
β-carotene bleaching inhibition (EC_{50}; mg/mL)	0.21 ± 0.02	Cinnamic acid (μg/g dw)	3.96 ± 0.16		
TBARS inhibition (EC_{50}; mg/mL)	0.06 ± 0.02				

EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the reducing power assay. GAE means gallic acid equivalents. Concerning the total phenolics assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, what means that lower values correspond to higher reducing power or antioxidant potential.

Table 4. Growth inhibition percentage (mean ± SD) of *Penicillium verrucosum* var. *cyclopium* in cream cheese incorporated with *Agaricus bohusii* extract, stored at room temperature and in refrigerator.

Conc./ Temp	10 mg/mL		20 mg/mL		40 mg/mL		60 mg/mL		80 mg/mL		100 mg/mL	
	+4°C	+25°C	+4°C	+25°C	+4°C	+25°C	+4°C	+25°C	+4°C	+25°C	+4°C	+25°C
0 h	0.0±0.0	0.0±0.0	0.00±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2. day	0.0±0.0	46.7±14.5	0.0±0.0	23.3±11.7	0.0±0.0	43.3±3.4	0.0±0.0	33.3±12.0	0.0±0.0	3.3±1.9	0.0±0.0	16.7±3.4
4. day	53.3±15.0	100.0±0.0	26.7±12.0	100.0±0.0	13.3±3.4	100.0±0.0	83.3±9.4	33.3±12.0	0.0±0.0	26.7±4.7	0.0±0.0	100.0±0.0
7. day	83.3±8.4	100.0±0.0	90.0±4.7	100.0±0.0	80.0±6.7	100.0±0.0	83.3±10.7	100.0±0.0	86.7±10.2	100.0±0.0	86.7±3.9	100.0±0.0

Table 5. Acceptability scores given by panelist for cream cheese and cream cheese + *A. bohusii* extract

	Cream cheese	**Cream cheese + extract**
Acceptibility*	4.1	4.8

*The results are expressed as the average of all grades.
1 = extremely dislike, 2 = dislike, 3 = neither like nor dislike, 4 = like; 5 = extremely like.

Figure 1. *Agaricus bohusii* Bon (Kibby, 2007).

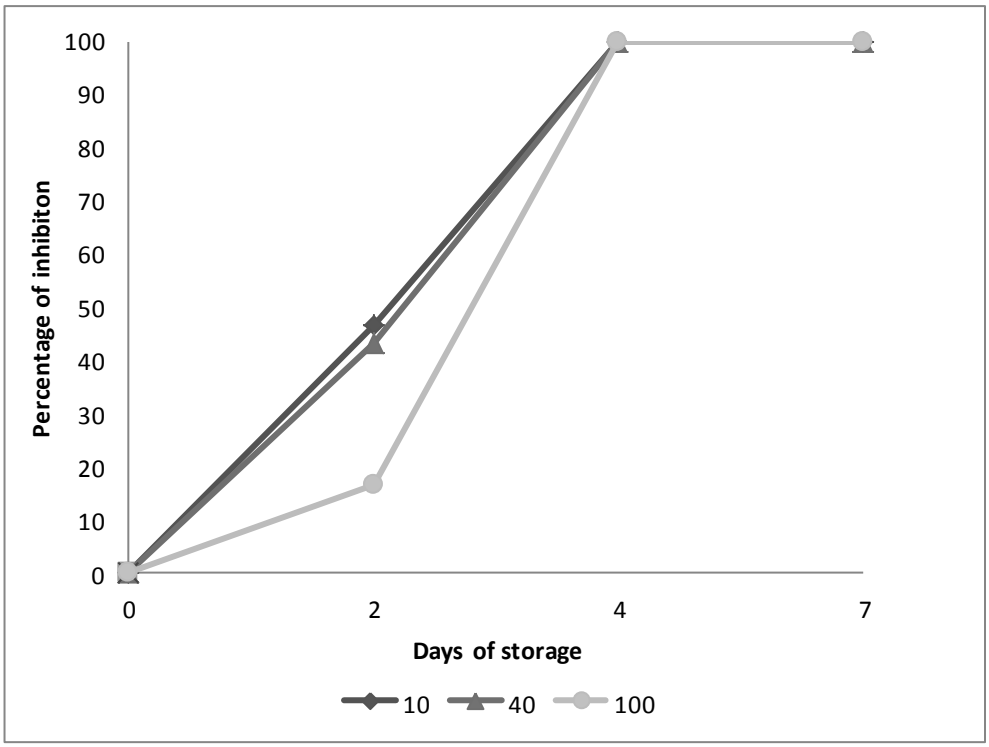

Figure 2. Inhibition percentage of *P. verrucosum* var. *cyclopium* with 10 mg/mL, 40 mg/mL and 100 mg/mL of *A. bohusii* methanolic extract during days of cream cheese storage at 25°C.